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Research Article

Cancer Prevention Research

The Detection of Chromosomal Aneusomy by Fluorescence *In situ* Hybridization in Sputum Predicts Lung Cancer Incidence

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Abstract

Lung cancer usually is disseminated (advanced) and has a poor prognosis at diagnosis. Current and former smokers are at a high risk for lung cancer and are candidates for prevention and early detection strategies. Sputum is a potential source of biomarkers that might determine either lung cancer risk or the presence of early lung cancer, but no current sputum test is sufficiently sensitive and specific for effective screening. We used fluorescence *in situ* hybridization (FISH) to measure chromosomal aneusomy (CA) in sputum samples collected prospectively from 100 incident lung cancer cases and 96 controls (matched on age, gender, and date of collection) nested within an ongoing high-risk cohort. The CA-FISH assay was aimed at four DNA targets: epidermal growth factor receptor, *MYC*, 5p15, and CEP 6. The sensitivity of a positive CA-FISH assay (abnormal for two or more of the four markers) for lung cancer was substantially higher for samples collected within 18 months (76% sensitivity) than for samples collected more than 18 months (31%) before lung cancer diagnosis. Sensitivity was higher for squamous cell cancers (94%) than for other histologic types (69%). CA-FISH specificity based on samples collected within 18 months before diagnosis was 88%. The adjusted odds ratio (OR) of lung cancer for specimens collected within 18 months before a cancer diagnosis was higher for the CA-FISH assay [OR, 29.9; 95% confidence interval (95% CI), 9.5-94.1] than for previously studied ORs of cytologic atypia (OR, 1.8; 95% CI, 1.3-2.6) and gene promoter methylation (OR, 6.5; 95% CI, 1.2-35.5). Whether CA-FISH is an indicator of extreme risk for incident lung cancer or detects exfoliated cancer cells is unknown. The apparent promise of CA-FISH in sputum for assessing lung cancer risk and/or for lung cancer early detection now needs to be validated in a clinical screening trial. *Cancer Prev Res; 3(4); 447–53. ©2010 AACR.*

Introduction

Survival rates for lung cancer have been low for many years (1) largely because the majority of patients have advanced-stage disease at diagnosis. The general risk for lung cancer can be estimated by several factors including smoking history, age, previous tobacco-related malignancy, family history of lung cancer, and the presence of chronic obstructive pulmonary disease (2–5). More precise factors, including better biomarkers, are needed, however, for identifying subgroups at a high enough risk to justify chemoprevention and/or early-detection interventions (6–9). Computerized tomography (CT) screening shows promise for early lung cancer detection, but its efficacy in reducing lung cancer mortality is not yet shown (10–14). If effective at all, CT screening likely will be more effective for highest-risk individuals; it also is likely to be more effective for peripheral than for central lung cancers, and so complementary methods for detecting cancers arising in the central airways could be helpful.

We have an ongoing program of prospective study of the ability of various biomarkers in sputum to predict and/or detect lung cancer. Our previous research showed that both moderate and higher levels of cytologic atypia (15–17) and gene promoter methylation (18, 19) could predict incident lung cancer in a cohort of heavy smokers with airflow obstruction and no history of cancer. We also showed that a four-target fluorescence *in situ* hybridization

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(FISH) assay was suitable for detecting abnormal cells in sputum samples from a small set of cases and controls nested within our ongoing cohort (20). Here, we report the lung cancer sensitivity and specificity of chromosomal aneusomy (CA) detected by the same four-target FISH assay in sputum samples from a far larger group of subjects.

Materials and Methods

Subject population

Subjects included in this study were enrolled in the Colorado High-Risk Cohort Study from 1993 to 2003. Methods of accrual and processing of specimens have been described previously (21) and are briefly described here. Subjects were recruited from community and academic pulmonary clinics, primarily in the metropolitan area of Denver, Colorado. At the time of enrollment, all subjects had a cigarette smoking history of at least 30 pack-years and significant pulmonary airflow obstruction but no history of cancer or acute respiratory infection. Cohort members were followed by active methods, including telephone and mail, and by passive methods including matching to the Colorado Department of Public Health and Environment Vital Statistics and Central Cancer Registry records. These patients were asked at study entry to collect two consecutive 3-day, early-morning, spontaneous cough sputum specimens in jars containing Saccomanno's fixative (2% carbowax and 50% alcohol), and most provided samples within 6 mo of enrollment. Repeat samples were then requested on an annual basis, although many cohort members provided samples only sporadically. Sputum specimens were stored at room temperature in the Tissue Procurement Core of the University of Colorado Specialized Program of Research Excellence in Lung Cancer.

In 2005, 114 incident lung cancer cases were identified and matched with 114 control subjects from the same cohort; the controls had never had lung cancer and were clinically cancer-free for at least 5 y following cohort enrollment. The individual matching was done according to gender, age (±6 y), and date of enrollment in the cohort study (±6 mo). FISH assay results could be obtained from 100 of these cases (88%) and 96 of these controls (84%). Stage was defined according to the Surveillance Epidemiology and End Results Summary Stage 1977 guidelines and the information was obtained directly from the Colorado Central Cancer Registry. Stage I was defined as localized, stages II and IIIA as regional, and stages IIIB and IV as distant. There was a single case of carcinoma *in situ* in the analysis that was grouped with the unknown category following the criteria previously used (15).

Assay methods

For FISH assays, specimens were processed according to a protocol previously described (20, 22) using ~300 μL of sputum from the same pooled sample collected from days 4 to 6 from which the cytologic examination was done.

Table 1. Characteristics of incident lung cancer cases and controls nested within the Colorado High-Risk Cohort

Characteristics	Cases (*n* = 100)	Controls (*n* = 96)
	n (%)	*n* (%)
Gender		
Male	75 (75)	71 (74)
Female	25 (25)	25 (26)
Race		
White	95 (95)	92 (96)
Other	5 (5)	4 (4)
Age (y)		
<65	37 (37)	35 (36)
≥65	63 (63)	61 (64)
Mean age (SD)	67.1 (8.04)	67.1 (8.20)
Smoking		
Current smoker	40 (40)	31 (32)
Former smoker	57 (57)	63 (66)
Current status unknown	3 (3)	2 (2)
Mean pack-years (SD)	74.2 (35.6)	66.9 (29.0)

The cells were washed two or three times in PBS and centrifuged, and the cell pellet was then incubated for 20 min in 1.5 mL HBSS/10 mmol/L EDTA/20 mmol/L DTT. After centrifugation, the pellet was then fixed in 1.5 mL of fresh methanol/glacial acetic acid and the cell suspension dropped within an ~12-mm-diameter circle drafted with a wax pen onto two uncoated, dry microscope slides. The slides were then air-dried and stored in a desiccator overnight, and one of them was selected for the FISH assays based on cellularity, nuclear morphology, lack of purulence, debris, and residual cytoplasm.

The FISH assays were done with the LAVysion probe set (Abbott Molecular) including one centromeric sequence (6p11.1-q1 1, CEP 6, labeled with SpectrumAqua) and three single copy DNA sequences: 5p15.2 (markers D5S23 and D5S721, labeled with SpectrumGreen), 7p12 (*EGFR*, labeled with SpectrumRed), and 8q24.12-q24.13 (*MYC*, labeled with SpectrumGold). The hybridization was done in freshly prepared slides (1-3 d old) after incubation in saline sodium citrate buffer (2× SSC) at 37°C for 30 min and in 70% glacial acetic acid for 1 min, digestion in pepsin (0.5 mg/mL in 10 mmol/L HCl) at 37°C for 10 min, and fixation in 1% formaldehyde at room temperature for 5 min. Seven microliters of the LAVysion probe were applied on the selected hybridization area, which was covered with a 15-mm circular glass coverslip and sealed with rubber cement. Denaturation and hybridization were done using the HYBrite thermocycler (Abbott Molecular) programmed for a 2-min melting time at 72°C and a 24-h hybridization at 37°C. Posthybridization washes included sequential incubations in 2× SSC/0.1% tergitol (nonylphenoxypolyethoxyethanol, NP40) for 2 min at

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73°C and in 2× SSC for 2 min at room temperature. Subsequently, slides were briefly dehydrated in ethanol and air-dried, and 4′,6-diamidino-2-phenylindole (0.3 μg 4′6′-diamidino-2-phenylindole dihydrochloride/mL of Vectashield mounting medium) was applied to counterstain the chromatin and protect from light exposure.

Analysis was done using epifluorescence microscopes equipped with single band pass filters for 4′,6-diamidino-2-phenylindole, Texas red, FITC, SpectrumAqua, and SpectrumGold. For documentation, images were acquired with a cooled charge-coupled device camera (Photometrics) in monochromatic layers and merged and processed using the CytoVysion software (Applied Imaging, Inc.). Selection of cells from analyses followed the FISH selective cell assessment previously described (20). The hybridization areas were scanned for putative epithelial cells with nonoverlapped, nondisrupted nuclei with distinctive signals. Sputum cells selected for analysis in the study had large, round or oblong-shaped nuclei with coarse chromatin texture and sometimes irregular nuclear borders. When detectable, the small ratio nucleus/cytoplasm was also an important criterion in the cell selection. The entire area (~12 mm diameter) was completely scanned by a trained cytogeneticist. On average, more than 500 nuclei were examined per slide and 30 nuclei were selected as cytologically "atypical" based on the morphologic features described above. Specimens carrying fewer than 100 nuclei with good hybridization signals were considered failures. Reasons for failure were insufficient cellularity in 300 mL of fixed cell suspension, degraded condition of cells, and unacceptable quality of hybridization in two attempts (a standard protocol plus customized troubleshooting).

The following criteria were used for assessment of the CA-FISH assay: An abnormal cell was defined as showing gain in copy number (more than two signals) for at least two DNA targets or gain in copy number for at least one DNA target and loss (less than two signals) for at least two DNA targets. Because tumor cells are expected to be rare in sputum, specimens were classified as abnormal when they showed ≥2% abnormal cells among the scored nuclei. A total of 401 sputum specimens blinded for the patient information (patient ID, time of collection and cancer status) were assayed by FISH, with an overall success rate of 83%. Success rate for patients was 86% (196 of 228 patients).

Statistical analyses

The sputum specimens were categorized into several subsets for independent investigation according to the number of months before the diagnosis of cancer in the cases (within 18 mo or longer than 18 mo before diagnosis) and according to the histologic type of lung cancer detected. In subjects with multiple specimens collected at a specific time frame, the specimen collected closest to the date of lung cancer diagnosis (or to the same date for the matched control) was the single sample used for these analyses. Sensitivity and specificity were estimated and logistic regression models were used to evaluate the association between lung cancer and FISH-detected CA with adjustment for potential confounding factors. Covariates

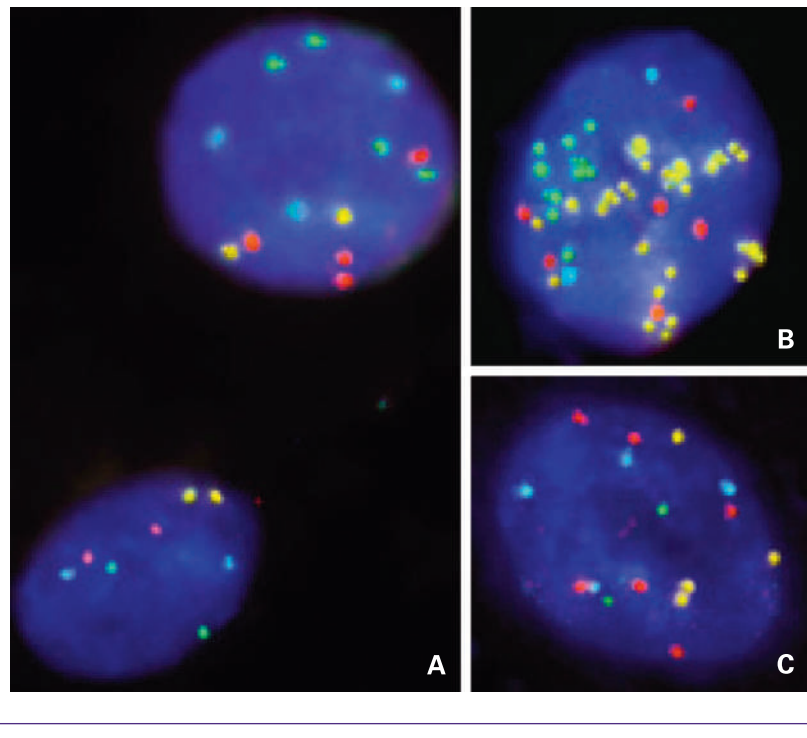

Fig. 1. Sputum specimens from three cases hybridized with the LAVysion probe set, including DNA sequences of epidermal growth factor receptor (*EGFR*; red), 5p15 (green), *MYC* (yellow), and CEP 6 (aqua). A, nuclei showing normal pattern (bottom left; 2 copies of red, green, yellow, and aqua signals) and abnormal pattern (4 copies of *EGFR*, 4 copies of 5p15, 2 copies of MYC, and 3 copies of CEP 6 signals). B, nucleus with copy number gain for all markers, including *MYC* gene amplification. C, nucleus showing copy number gain for three markers (*EGFR, MYC*, and CEP 6).

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Table 2. Abnormalities in selected and pooled FISH aneuploidy markers among nested incident lung cancer cases and controls sampled from the Colorado High-Risk Cohort

Marker	Cases* (n = 100)	Controls* (n = 96)	OR (95% CI)	
	Abnormal	Abnormal	Age-adjusted model	Fully adjusted model*
	n (%)	n (%)		
Individual DNA markers				
EGFR	59 (59)	21 (22)	5.2 (2.8-9.8)	4.9 (2.6-9.4)
5p15	47 (47)	13 (14)	5.7 (2.8-11.5)	5.6 (2.7-11.4)
MYC	46 (46)	12 (13)	6.0 (2.9-12.4)	5.5 (2.6-11.5)
CEP 6	19 (19)	2 (2)	11.1 (2.5-49.0)	12.0 (2.7-54.5)
No. of abnormal markers among the pooled four				
None	31 (31)	67 (70)	1.0 (reference)	1.0 (reference)
Any of the four	69 (69)	29 (33)	5.2 (2.8-9.4)	5.0 (2.6-9.3)
≥2 markers	56 (56)	14 (15)	8.8 (4.2-18.2)	8.3 (3.9,17.7)

Abbreviation: 95% CI, 95% confidence interval.
*Adjusted for age, sex, FEV1, pack-years of smoking, and current smoking status.

included age (continuous), sex, smoking status (former versus current at baseline), FEV1, and pack-years of tobacco use. Former smokers were defined as having quit at least 1 y at the time of enrollment. Pack years were defined as the average number of packs smoked per day multiplied by the number of years smoked. The two variables comprising pack-years were those reported at time of enrollment. Associations were expressed as odds ratios (OR) with corresponding 95% confidence intervals. Univariate and multivariate models adjusting for covariates were implemented. All analyses were carried out using Statistical Analysis Software (version 9.2, SAS Institute, Inc.).

Table 3. Abnormality in FISH, defined as 2 or more markers abnormal among the four tested markers, by time between sputum collection and the date of lung cancer incidence, by cancer histology, and by cancer stage

Characteristic	Cases		OR (95% CI)*	
	n/N abnormal	%	Age-adjusted model	Fully adjusted model†
Time between sputum collection and lung cancer incidence				
More than 18 mo	14/45	31	2.0 (0.7-5.6)	1.7 (0.6-5.3)
Within 18 mo	42/55	76	24.1 (8.8-65.8)	29.9 (9.5-94.1)
Among samples collected within 18 mo before diagnosis				
Lung cancer histology				
Squamous cell	15/16	94	131.1 (13.6-999)	Not estimable
Adenocarcinoma	11/17	65	12.9 (3.6-46.2)	16.4 (3.7-72.2)
Small cell	3/5	60	10.2 (1.4-73.7)	4.7 (0.2-99.9)
Others	10/13	77	29.8 (6.0-148.8)	77.8 (7.9-764.5)
Unknown	3/4	75	21.0 (1.7-254.4)	10.3 (0.4-242.4)
Lung cancer stage				
Localized (stage I)	14/15	93	123.5 (12.4-999)	Not estimable
Regional (stage II, IIIA)	4/7	57	12.2 (2.0-73.5)	15.7 (2.1,118.6)
Distant (stage IIIB, IV)	8/14	57	9.0 (2.4-34.7)	20.6 (3.6-118.0)
Unknown	16/19	84	39.8 (9.1-173.4)	37.7 (7.6-186.8)

*ORs comparing cases to the pooled control group, among whom there were 7 of 59 (12%) who had ≥2 abnormal FISH markers for ≤18 mo proximal samples and 7 of 37 (19%) who had ≥2 abnormal FISH markers for >18 mo proximal samples.
†Adjusted for age, sex, FEV1, pack-years of smoking, and current smoking status.

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Results

This study compared 100 cases with 96 controls (Table 1). The mean age was 67 years, and the majority of patients were male (75%), former smokers (63%), and white (96%). These cases involved sputum specimens collected either within 18 months or more than 18 months before a clinical diagnosis of cancer; the study focused on samples collected within 18 months of diagnosis. Figure 1 illustrates sputum cells with normal copy numbers for all analyzed DNA targets and with copy number gain for three or four of these targets.

There was a significant association between detection of CA and cancer status in cases or controls for each of the four FISH markers, but sensitivity for all the single markers ranged from as low as 19% to 59% (Table 2). Among all the samples, sensitivity based on two or more of the four markers being positive was 56% and specificity (the proportion of controls testing negative) was 85%. The age-adjusted and fully adjusted ORs increased from ~5 when any one of the four markers was abnormal to ~8 when two or more of the markers were abnormal.

A large difference in CA sensitivity depended on the time between sample collection and lung cancer diagnosis (Table 3). Sensitivity increased from 31% for samples collected more than 18 months to 76% for samples collected within 18 months before a cancer diagnosis. Among samples collected within 18 months of diagnosis, sensitivity was 95% for cases diagnosed with squamous cell cancer and 93% for cancers diagnosed at a localized stage (Table 3). Sensitivity and specificity overall and by histology were slightly improved for samples collected within 12 months (Supplementary Table S1) or 6 months before diagnosis, but the number of specimens in the 6-month interval was small enough to prevent stable analysis.

Abnormalities detected by FISH were associated significantly with cytologic atypia (Kendall's $\tau = 0.44$; $P < 0.001$); a higher frequency of abnormal specimens was detected by FISH than by cytology among cases (Supplementary Table S2). Receiver operating characteristic curve analyses showed that the area under the curve (AUC) was highest when FISH and cytology were in the model (AUC = 0.85) but not significantly better than FISH alone (AUC = 0.84; Supplementary Fig. S1). Both models have included adjustments for age, sex, FEV1, pack-years of smoking, and current smoking status. Furthermore, the association between cytologic atypia and individual tumor subtypes was overall lower than detected by FISH markers (Supplementary Table S3).

Discussion

Lung carcinogenesis biomarkers expressed in readily accessible samples such as blood (23), sputum (8), large-airway epithelial cells obtained at bronchoscopy (24), exhaled breath (25), or urine (26) have great potential for identifying subgroups of people with an extreme lung cancer risk who might benefit from chemoprevention, for lung cancer early detection, and for informing clinical decisions in the setting of CT-detected lung nodules. Although under active investigation, no such biomarkers have been validated for clinical application. A recent study found that matched

Table 4. Comparison of CA FISH findings in this analysis with previously published findings for sputum cytology and sputum DNA methylation markers from this same cohort

	Cases, *n/N* abnormal	Sensitivity	Specificity	Fully adjusted OR (95% CI)
All samples from previous reports and the current report				
Cytologic atypia*	50/174	29%	83%	1.8 (1.3-2.6)
DNA methylation†	33/52	64%	64%	6.5 (1.2-35.5)
CA-FISH‡	42/55	76%	88%	29.9 (9.5-94.1)
Subset of samples on which all three biomarkers were performed				
Cytologic atypia	20/48	42%	77%	2.3 (0.8-6.4)
DNA methylation	33/48	69%	54%	4.3 (1.0-19.8)
CA-FISH	37/48	76%	85%	27.2 (7.8-94.1)

*From Ref. 15. Atypia is defined as moderate atypia or worse. OR is adjusted for age, sex, race/ethnicity, pack-years of smoking, smoking status at baseline, and year of enrollment into the cohort. Among controls, there were 400 of 2,347 (17%) who had ≥2 abnormal FISH markers for ≤18 mo proximal samples.

†From Ref. 18. Methylation is defined as three or more of a panel of six markers methylated. OR is adjusted for age, sex, FEV1, pack-years of smoking, and current smoking status. Among controls there were 17 of 47 (36%) who had ≥2 abnormal FISH markers for ≤18 mo proximal samples.

‡Data from the current report. Positive FISH assay is defined as two or more of four FISH markers being abnormal. OR is adjusted for age, sex, FEV1, pack-years of smoking, and current smoking status. Among controls, there were 7 of 59 (12%) who had ≥2 abnormal FISH markers for ≤18 mo proximal samples.

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sputum and tumor tissues from lung cancer patients carried similar chromosomal deletions (27). Chromosomal missegregation, with resulting losses and gains, is a hallmark of cancer, and CA in the present study was strongly associated with lung cancer incidence in samples collected prospectively within 18 months before lung cancer diagnosis. There was very little CA-lung cancer association, however, for samples collected more than 18 months before diagnosis. Therefore, the CA-FISH biomarker abnormality is either detecting exfoliation of cancer cells into sputum or detecting serious field effects that occur very late in the development of lung cancer. This CA assay has sufficient sensitivity (76%) and specificity (85%) to be potentially useful in selected clinical screening settings.

Results of the present nested case-control study build on those of our previously published investigation of CA (20) by tripling the number of subjects from 66 (33 case-control pairs) to the 196 reported here. The results of the present study in a single sample collected within 18 months of diagnosis are generally consistent with the previous findings for samples collected within 12 months of diagnosis (20). Of importance, the present CA-FISH assay had greater sensitivity (76%) and specificity (88%) than did either cytology (29% sensitivity, 83% specificity) or gene promoter methylation (64% sensitivity or specificity) in our previous studies (Table 4; refs. 15, 18). Although all assayed in the same cohort, these biomarkers were examined in different subsets of that cohort and so the comparisons among them are not prospective. Therefore, we compared the CA-FISH, atypia, and methylation results of the same subset of 48 cases and 48 controls who each gave a single sample that underwent all three assays, and found the results to be very similar to those of the nonprospective comparisons (Table 4).

We believe that CA-FISH deserves further study in other high-risk populations to validate its utility as a primary screening test for lung cancer or lung cancer risk assessment or, given the strong performance of CA in detecting early-stage (localized or regional) disease, its utility in assisting the clinical management of patients already suspected to have cancer. Although the strong CA-FISH performance was independent of cytologic atypia and promoter methylation, further analyses of the independence and complementariness of all three approaches in larger study populations are needed to determine whether combining CA and the other biomarkers will improve sensitivity and/or specificity for the early detection or precise definition of the risk of lung cancer. A recent analysis found that a combined screening approach with CT scans and FISH probes for deletions in *HYAL2*, *FHIT*, *p16*, and *SP-A* in sputum was more sensitive, specific, and accurate for diagnosing central lung cancers than was CT alone (28).

It is somewhat surprising that the CA assay in expectorated sputum was so highly specific for lung cancer because it had a lower specificity in studies of our and other groups in premalignant respiratory epithelium (29). We speculate that the process of exfoliation itself may be partially responsible for the increased specificity we saw in the present study.

This study provides a proof of principle for CA-FISH screening for lung cancer risk and early detection. The favorable sensitivity, especially for squamous cell cancer and localized, early disease, supports further study to validate the assay. The specificity of CA-FISH exceeds that of any other sputum test reported to date, and the performance characteristics likely can be further improved. The LAVysion set of four probes may not be optimal for FISH detection of lung cancer. Ongoing research is comparing this original probe set with others based on commonly amplified chromosomal regions in lung cancer (30–34). Compared with deletions, amplifications are less likely to occur as an artifact in the preparation of samples and thus are more desirable targets for FISH cocktails.

Although the current CA-FISH test is labor-intensive, time-consuming, and may not be ready for immediate clinical application, the promise of our current results supports efforts to simplify the assay. Obvious areas to explore for simplification include a higher degree of automation, techniques to enrich the number of epithelial cells in the sample, and combining FISH with immunohistochemical markers to more readily identify the epithelial target cells. Small recent studies have shown some promising results in these areas (35, 36).

The CA-FISH assay has a number of limitations. Sensitivity decreases with time between sputum-sample acquisition and cancer diagnosis, and so this test seems to mark lung cancer itself and/or very severe but late field changes of lung premalignancy rather than long-term lung cancer risk. The assay is less sensitive for adenocarcinoma than for squamous cell lung cancer. However, because central airway neoplasms, which generally are squamous cell, are less likely to be detected by CT scan, CA-FISH may well complement CT screening in the diagnosis of squamous cell cancers and potentially may be an aid to decision making in the clinical management of lung nodules.

In conclusion, the CA-FISH assay was more sensitive and more specific for predicting lung cancer incidence than was cytology or DNA methylation. CA-FISH identifies chromosomal missegregations, a hallmark of carcinomas and an established molecular feature of advanced lung premalignancy. It is a promising tool for identifying the highest-risk individuals within more general risk groups (e.g., chronic smokers) and for detecting early lung cancer before its clinical manifestation. CA-FISH should be further evaluated in lung cancer screening trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

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