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Perspective

Cancer Prevention Research

Lung Cancer Biomarkers: FISHing in the Sputum for Risk Assessment and Early Detection

Perspective on Varella-Garcia et al., p. 447

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Abstract

This perspective on Varella-Garcia et al. (beginning on p. 447 in this issue of the journal) discusses the role of sputum-based biomarkers in the risk assessment and early detection of lung cancer. The importance of the detection of sputum epithelial chromosomal aneusomy by fluorescence *in situ* hybridization (FISH) as a potential risk or early-detection biomarker is discussed in the context of other biomarkers and models in lung carcinogenesis. The presently reported findings on FISH in sputum cells are an important contribution worthy of further investigation in defined clinical settings. *Cancer Prev Res; 3(4); 420–3. ©2010 AACR.*

Lung cancer is the leading cause of cancer death in both men and women in the United States and causes more than 1 million deaths worldwide per year (1, 2). There are more than 100 million current and former smokers in the United States who have an elevated risk for lung cancer (3). Clinicians commonly encounter current and former smokers suspected to have lung cancer on the basis of an abnormal imaging study, and the need for effective noninvasive methods to confirm the presence of early lung cancer in these high-risk individuals is compelling. Furthermore, methods to define those at the highest risk of developing lung cancer could provide a window of opportunity for chemoprevention. One noninvasive approach that may significantly improve the assessment of risk, early diagnosis, and application of chemoprevention for lung cancer is to assess molecular biomarkers in noninvasively obtained biospecimens.

Although high-risk profiles exist for heavy smokers (50 years or older, 30 or more pack-years of smoking history, emphysema, and asbestos exposure), no models that incorporate biomolecular markers of risk have been validated. Patients at risk for lung cancer may have subclinical disease for years before presentation and diagnosis (4). The clinical challenge, therefore, is to develop effective lung cancer risk assessment models for use during this window of opportunity in identifying highest-risk patients for chemopreventive investigation and for increasing the rate of early detection and thus early treatment. Complicating the pathway to discovery in this area is the possibility that risk-assessment and early-detection markers may differ. Furthermore, analogous to advanced-stage lung adenocarcinoma, where heterogeneity in driver mutations seems to define multiple different disease states, interindividual molecular heterogeneity in the pulmonary field at risk may lead to subsets of risk biomarkers that reflect differences in mutational events in high-risk premalignancy.

Growing biomarker data show that cigarette smoking creates a field of injury in all exposed airway epithelial cells (5–11). A major result of this injury is inflammation and its role in lung carcinogenesis. For example, *K-ras* mutations and/or p53 loss can induce interleukin-8, cyclooxygenase-2, and nuclear factor κB activation, which results in changes in the pulmonary microenvironment, including changes in chemokine levels, that are important in the progression of inflammation and lung carcinogenesis (12, 13). Interest in the role of telomeres and telomerase activity within the inflammation-carcinogenesis link is growing (14, 15). Recent data indicate that alterations in telomerase expression precede malignant transformation in the lung (16) and that polymorphisms in telomere maintenance genes (17) and shortened telomeres (18) are associated with an increased risk of lung cancer.

Sputum is an attractive potential source of biomarkers for lung cancer because it can be obtained noninvasively and may represent the field of injury. Consequently, many research groups have been examining sputum for potential biomarkers, but to date, there are no definitive data indicating that sputum biomarkers are sufficiently sensitive and specific to reliably predict which high-risk patients will go on to develop lung cancer.

Early lung cancer–related studies of sputum examined cytologic atypia. Two randomized controlled trials of lung

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cancer screening, the Johns Hopkins Lung Project and Memorial Sloan-Kettering Lung Study, were initiated in the 1970s (19). They compared annual chest X-ray (CXR) plus 4-monthly sputum cytology (dual screen) with annual CXR only. Previous publications from these trials based on incomplete follow-up revealed no difference in mortality between the two groups, but each trial on its own was underpowered. An intention-to-screen analysis of lung cancer mortality used combined data from both trials ($n > 20,000$) and showed that more than 50% of squamous cell lung cancers diagnosed in the CXR-plus-cytology group were identified by sputum cytology. After 9 years of follow-up, lung cancer mortality was slightly lower in the dual-screen than in the CXR-only arm, and these reductions were seen in squamous cell cancer deaths and in the heaviest smokers (19). The differences between groups were small, however, and could have occurred by chance.

Fan et al. (20) conducted a large prospective cohort study of sputum cellular atypia in occupational tin miners in Yunnan, China, based on data from an annual lung cancer screening program. Sputum samples were collected prospectively at baseline and annually for 7 years. Cytologic atypia in sputum was an independent risk factor for lung cancer. The authors concluded that sputum cytologic examination combined with other screening approaches could play an important role in the early detection of lung cancer or in the selection of the optimal target population for more intensive lung cancer screening in this high-risk occupational cohort (20).

It has been suggested that combined-modality screening may be effective for identifying early-stage lung cancer and very high-risk populations for close follow-up. Lam et al. (21) followed 181 patients at a high risk for lung cancer (40-or-more-year-old ever smokers with a history of more than 20 pack-years). Patients with sputum atypia (47%) underwent bronchoscopy and computed tomography (CT) scans of the chest. Primary lung cancer was diagnosed in 15% of these patients, almost half of which were diagnosed at early stage. Nearly all of the sputum atypia group had radiologic abnormalities, although three lung cancers (all stage 0) were missed by CT. Among a further 6% of patients who developed primary lung cancer during follow-up, only 20% were detected by sputum atypia. The overall sensitivity of sputum cytology in detecting lung cancer was 71% for all histologies and 100% for squamous cell lung cancer (21).

Further studies have attempted to improve the sensitivity and specificity of sputum cytology by concentrating and purifying the exfoliated bronchial epithelial cells in sputum and removing populations of other cell types. Qiu et al. (22) used magnetic-assisted cell sorting with anti-CD14 and anti-CD16 antibody beads to deplete macrophages and neutrophils from sputum. Fluorescence *in situ* hybridization (FISH) analysis for detection of fragile histidine triad (*FHIT*) deletion and cytology were done in the enriched specimens. They were able to increase the bronchial epithelial cell concentration from 1% in the starting population to 40% in the enriched population. Detecting *FHIT* deletions led to a sensitivity of 58% for lung cancer detection in the enriched sputum, in contrast to a sensitivity of 42% in the un-enriched samples ($P = 0.02$). Enrichment also significantly improved the sensitivity of cytologic examination ($P = 0.03$; ref. 22). Katz et al. (23) used an automated quantitative system to score 3p22.1 and 10q22.3 (*SP-A*) genetic abnormalities by FISH analysis in epithelial cells from noninduced sputum specimens; the FISH abnormalities were examined for correlation with cellular morphology. Epithelial cells in sputum from patients with non–small-cell lung cancer were cytologically and genetically abnormal compared with cells from a high-risk or healthy control group. The investigators developed a model to assess the risk of lung cancer in each study patient. Although this was a small study, the authors predicted that the best sputum assay for lung cancer would combine assessments of morphologic characteristics and molecular abnormalities in both atypical cells and morphologically normal cells (23).

Examining exfoliated bronchial epithelial cells for known genetic mutations in lung cancer has improved the value of sputum for lung cancer screening. Baryshnikova et al. (24) performed a large study to establish the frequency of *K-ras* and *p53* mutations and p16(INK4A), RASSF1A, and NORE1A hypermethylation in sputum of a large cohort of cancer-free heavy smokers and to assess whether these markers could be useful for clinical screening for lung cancer early detection. This study led to the following results: 7% of the cohort had one of these molecular alterations; *p53* mutation and p16(INK4A), RASSF1A, and NORE1A methylation frequencies were 1.9%, 5.1%, 0.8%, and 1.0%, respectively; no *K-ras* mutations were found. Thus far, however, sufficient follow-up has not been reported to determine the predictive value of these genetic and epigenetic alterations in sputum in this cohort. Xie et al. used real-time reverse transcription-PCR to show another meaningful epigenetic change: the expression of mir-21 was significantly greater in sputum from 23 patients with non–small-cell lung cancer than in sputum from 17 cancer-free control subjects. Detection of mir-21 expression produced 69% sensitivity and 100% specificity in diagnosing lung cancer, compared with 48% sensitivity and 100% specificity with sputum cytology (25).

Investigators are now using combined genetic analysis of sputum and CT scans to screen for early lung cancer. Because CT scanning may have a limited capacity to detect very early lesions, especially in centrally located areas, it has been suggested that combined sputum genetic assays and CT might improve screening detection of very early disease. Jiang et al. (26) analyzed genomic copy changes of *HYAL2*, *FHIT*, *p16*, and *SP-A* by a mini-chip in sputum from 33 patients with stage I non–small-cell lung cancer and from 49 cancer-free controls. The genetic changes and CT scan diagnoses were compared with the surgical-pathologic stage. CT had a higher sensitivity (85%) for detecting lung cancer compared with the mini-chip genetic changes (70%; $P < 0.05$), and there was no significant difference in specificity between the two tests (89% versus

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92%; $P = 0.09$). CT also showed considerably higher sensitivity (93%) in identifying peripheral tumors than did the mini-chip (64%; $P < 0.05$), whereas there was no difference in specificity for this subtype between the two tests (98% versus 96%; $P = 0.28$). In detecting central tumors, however, CT had a lower specificity (90%) compared with the mini-chip (98%; $P < 0.05$), although its sensitivity (79%) was somewhat higher than that of the mini-chip (73%; $P = 0.05$). Combining both tests offered higher sensitivity (91%) than did either one alone in any setting (85% and 70%; all $P < 0.05$) and maintained 92% specificity sensitivity (26). An analysis in sputum specimens from a small number of patients also recently found that deletion detected by a panel of the four FISH probes, *HYAL2*, *FHIT*, *p16*, and *SP-A*, improved the sensitivity, specificity, and accuracy of CT scans for diagnosing central lung cancers (26).

In this issue of the journal, Varella-Garcia et al. (27) report that chromosomal aneusomy in sputum is a promising noninvasive biomarker for assessing lung cancer risk among heavy smokers with chronic obstructive pulmonary disease. In work that advances the field, the authors showed that chromosomal aneusomy was strongly associated with lung cancer incidence for samples prospectively collected within 18 months before a lung cancer diagnosis. They used the chromosomal-aneusomy FISH (CA-FISH) assay, which identifies chromosomal missegregations, a hallmark of carcinomas and a molecular feature of premalignant lung lesions (28). This study compared 100 cases with 96 controls, and the CA-FISH assay sensitivity and specificity were 76% and 85%, respectively. The CA-FISH assay was more sensitive and specific for predicting lung cancer incidence than was cytology or DNA methylation in sputum samples in previous small studies by this group (29, 30).

In accord with previous investigations, Varella-Garcia et al. found the CA-FISH assay to be more sensitive for central-airway tumors. These findings support the hypothesis that sputum-based assays reflect disease of the central airways and have more limited value for the bronchioloalveolar compartment. Therefore, this test may complement CT scanning and may assist in diagnosing indeterminate nodules. The sensitivity of the assay correlated strongly with the time of sample acquisition; specimens obtained more than 18 months before diagnosis were of less value. This provocative finding suggests that many study subjects had lung cancer at the time of evaluation. Further studies will be required to determine if chromosomal aneusomy by FISH can delineate populations for chemoprevention. Such a chemoprevention window for high-risk subjects would constitute a major advance in the field. Another interesting finding in this study was that the positivity rate for localized disease was higher than that for distant/regional disease (14/15 versus 12/21; $P = 0.03$). This result suggests that the CA-FISH assay may be useful for early detection, but the biological mechanism underlying this finding is unclear.

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. The study was designed to improve the sensitivity of risk assessment by also performing genetic testing with FISH analysis of cells in the sputum. The FISH analysis was associated with a failure rate of 14%. Cytologic atypia was found to be not as useful as FISH assay of sputum cells for determining the risk of lung cancer in heavy smokers with chronic obstructive pulmonary disease. Combining both tests did not increase specificity and sensitivity over those of the FISH markers alone. With a specificity of 85%, this study's FISH markers in sputum specimens collected within 18 months of clinical diagnosis exceeded the specificity of any other prospective lung cancer screening sputum test reported to date. Current research is comparing this original probe set to others based on chromosomal regions commonly amplified in lung cancer (20, 24, 26). In future studies, evaluation of additional chromosomal abnormalities, application of techniques for epithelial cell enrichment, and new methods for automated sputum assessment could allow for better scrutiny of abnormal cells and facilitate translation into clinical settings. These are additional features that may all contribute to augmenting the performance of this assay (20, 24, 26, 31).

The message of the substantial increase in risk associated with positive FISH markers within 18 months may be that these markers are potentially early-detection rather than risk markers. The percentage of existing (but undetected) tumors was likely much higher in the group that gave sputum samples within 18 months of diagnosis than in the greater-than-18-months group. Risk markers [e.g., the exposure and demographic measures assessed by Spitz et al. (32)] would likely show a more constant prognostic effect over time. Therefore, these new findings suggest that this FISH assay may work at the clinical interface of early detection with prevention (33). Such early detection marks the convergence of therapy with prevention, or preventing microscopic cancer from developing into clinical disease.

The work of Varella-Garcia et al. (27) seems to support a specific aspect of the risk equation, the risk of centrally located lung cancer in heavy smokers. Still to be defined is risk assessment or detection strategies for peripheral disease and nonsmokers. Ohashi et al. (34) recently showed the potential utility of molecular-targeted chemoprevention against non–smoking-related lung cancer in a murine model engineered to express EGFR L858R in type II pneumocytes. These findings are consistent with previously published findings by the Wistuba group, who detected *EGFR* tyrosine kinase domain mutations in the histologically normal respiratory epithelium of lung cancer patients (35). Therefore, recent studies of mice, clinical specimens, clinical-epidemiologic data, and environmental risk factors suggest new opportunities for informed chemoprevention in the never-smoker population (36) that could be explored in response to the "call to action" regarding lung cancer in this population (37). This new area of chemoprevention will require close interaction with new and developing biostatistical methods.

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In conclusion, formidable barriers remain for the development of noninvasive assessments of lung cancer risk and early detection with high sensitivity and specificity. Varella-Garcia et al. have made an important contribution worthy of further investigation in defined clinical settings. Their FISH-based sputum assay may be useful in assessing lung cancer risk among heavy smokers with chronic obstructive pulmonary disease and in assisting in the diagnosis of indeterminate pulmonary nodules defined by imaging. Furthermore, cytologic and FISH changes may help identify an individual's clonal premalignant changes in the field of "cancerization" that could lead to the identification of novel targets and personalized chemoprevention.

Disclosure of Potential Conflicts of Interest

A. Spira is consultant to and has equity in Allegro Diagnostics, Inc. S.M. Dubinett serves on the scientific advisory board of Tragara Pharmaceuticals. The other authors declared no potential conflicts of interest.

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