

DIAGNOSIS OF ROENTGENOGRAPHICALLY OCCULT LUNG CANCER BY SPUTUM CYTOLOGY

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Cytologic detection of lung cancer in sputum can be traced to the 1930s, 1940s, and 1950s.²⁻¹¹ Dr. Papanicolaou, famed for his development of the pelvic Pap test, also described sputum cytology in the mid-1940s.³⁴ Other great leaders, such as the late Drs. Liebow, Farber, Bickerman, Barach, and Frost, described various clinical techniques for the detection of lung cancer.^{6,9,10,13,21}

One of the authors (TLP) was a first-year resident at the University of Michigan in 1959 when pathologist Walter Umiker announced at chest conferences that "two sputum cytologies were equal to one biopsy."⁴⁹ Later, the authors met Geno Saccomanno of Grand Junction, Colorado, the person who popularized, standardized, and studied sputum cytology more than any other person in the world.^{31,9} The late Saccomanno's atlas of Diagnostic Pulmonary Cytology is a collector's item.³⁶ In visionary articles introduced to the literature 2 decades ago,^{15, 17} Saccomanno's late colleagues, Don Greenberg and John Frost, also prophesied that sputum cytologic diagnosis one day would yield to automated methodology.

Papanicolaou and Saccomanno described the morphologic progression of cytologic atypia through progressive stages of dysplasia to squamous cancer. This epidermoid progression has

characterized roentgenographically occult central lesions, most of which are squamous cell carcinomas. Now, using molecular markers of neoplasia, all major lung cancer cell types have been detected by cells exfoliated into the sputum.^{33,45} A well-studied cohort of patients in Grand Junction has revealed that roentgenographically occult lung cancer identifies patients with a high percentage (more than 80%) of stage 0 or stage 1 cancer, whereas the actual 5-year cure by surgery in 27 cases was excellent.³ Subsequent cancers also were identified by sputum cytology or advanced imaging techniques in a follow-up of this cohort.⁴

Today, there is a growing interest in the early identification of asymptomatic lung cancer by CT scan imaging techniques. This interest is, in large part, the influence of the initial report of the Early Lung Cancer Action Project (ELCAP), which showed that smokers older than the age of 60 years had a prevalence of lung cancer of 2.7%, most of which were early stage lesions.⁹ ELCAP has led to a rethinking of the likelihood of cost-effective identification of lung cancer at an early stage (stage shifting) through screening.⁴¹ Early reports of helical CT scan screening, however, detected primarily (peripheral) adenocarcinomas and not the expected numbers of (central) squamous

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or small cell lung cancers. Detection of all lung cancer cell types and lesion locations suggests that sputum cytology and CT scanning are complementary screening techniques.

PATIENTS AT HIGH RISK FOR LUNG CANCER

Patients with air flow obstruction have a higher incidence of lung cancer than patients who have normal air flow with equal risk factors in terms of smoking, family history, and occupational risk.⁷ "• 26,40, 44 ^ g^y level of cigarette smoking, a four- to sixfold increased risk for developing lung cancer is observed when air flow obstruction is present.^{40,44}

This concept was bolstered by the Lung Health Study, in which the most common cause of death was lung cancer in young patients (aged 35 to 59), with only mild degrees of air flow obstruction.¹ A late follow-up of this cohort now has revealed a total of 227 cancers from an enrollment of 5887 subjects (3.9%) (John E. Connett, PhD, personal communication, 1999). The preliminary results of the Moffitt lung cancer screening study have shown a similar high lung cancer prevalence (3.4%: 20 cases or 579 screened) among air flow-obstructed, middle-aged, current and former heavy smokers (s forced expiratory volume in one second (FEV₁)/forced vital capacity 70% s 45 years old, s: "30 pack years").⁴³

This article discusses where sputum cytology should be used in the diagnosis and assessment of patients at risk for lung cancer or in the follow-up of patients with previous lung cancers, and the promising new sputum cytology techniques that soon may be introduced into the clinical practices of medicine.

SPUTUM COLLECTION AND HANDLING

Two of the authors (TLP, MST) evaluated the adequacy of spontaneous sputum collection at home or in the office after inhalation of hypertonic saline.²⁴ Both techniques of collecting sputum after inhalation of hypertonic saline and in-home collection after a hot shower produced equivalent results when dual tests were performed in random order. Sputum with the highest cell counts tended to be collected on the second occasion, suggesting a learning effect.²⁴ Home sputum collection has been used in a study among air flow-obstructed current smokers conducted by the Specialized Program of Research Excellence (SPORE) at the University of Colorado and has produced an approximate 2% yield of carcinoma in situ, invasive carcinoma, or high dysplasia, and 25% moderate dysplasia.²³ Many of these patients with moderate dysplasia were the subject of further examination by fluorescent endoscopy for small, centrally localized bronchial intraepithelial lesions. Six tiny, intraepithelial cancers have been found in 79 consecutive patients undergoing bronchoscopy because of the

presence of moderate dysplasia in expectorated sputum.²²

The techniques of sputum processing, slide making, staining, and analysis have undergone considerable revisions since Saccomanno's pioneering work. Sputum is a complex mixture of marrow-derived inflammatory and exfoliated airways epithelial cells accompanied by varying amounts of mucoid glycoprotein, serous elements, and saliva. Marrow-derived acute and chronic inflammatory cells may comprise up to 50% of the cell mix in an active smoker. The inflammatory cells can be ignored after noting the presence of alveolar macrophages to ensure the presence of adequate pulmonary (not merely oropharyngeal) sampling. Most sputum cellular elements are exfoliated airway epithelial cells in various stages of apoptosis. Like the colon, the airways epithelium constantly renews itself, and mature, squamoid intermediate cells with expansive cytoplasm and small, often pyknotic nuclei are contributed by upper and lower airways. These intermediate cells also are ignored routinely. The primary interest focuses on the 0.1% to 1% of epithelial cells with intact nuclei that can provide morphologic or molecular evidence of neoplastic evolution.

The accuracy of lung cancer screening by detection of morphologic preneoplastic changes in sputum epithelial cells was studied carefully 3 decades ago in the National Cancer Institute's Collaborative Early Lung Cancer Detection studies (at Memorial Sloan-Kettering, Mayo Clinic, and Johns Hopkins).¹⁴ Routine cytopathology was found to be highly specific (few false-positives) but relatively insensitive for lung cancer screening. In the experience of the Johns Hopkins Hospital, sputum cytology found only 22% of the lung cancers in the population at first screening (prevalence) and only 11% on repeat (incidence) screening.⁴⁶ Although mass screening of sputum for morphologic neoplastic changes provided a low yield, this technique is not without value. Routine sputum cytology examination still is recommended to assist an astute physician with individual case finding during evaluation of a patient at high risk for lung cancer.

Sputum preservation for routine morphology examination continues to follow the Saccomanno guidelines. The 50% ethanol, 2% polyethylene glycol-1450 solution used by Saccomanno remains a good way to preserve cellular morphology. Short DNA fragments and many proteins also may be preserved for years as a sputum slurry in Saccomanno's solution. Cellular RNA is lost almost immediately. Commercially available improvements in sputum cell preservation solutions and automated slide preparation are of value. For the best preservation of nucleotides, protein, and morphology, however, more extensive processing is needed. The H. Lee Moffitt Cancer and Research Institute in Tampa, Florida uses a laminar flow sputum induction hood in a small preparation area where the sputum immediately is suspended in a

dithiothreitol solution, washed in Hank's physiologic saline solution, spun, and suspended in EDTA.⁴⁸ After a second wash and resuspension, sputum to be preserved for nucleotide examination is aliquoted and placed in freezing medium (10% Dimethylsulfoxide (DMSO), 90%), similar to the standard method for viable freezing of tissue cultures in liquid nitrogen. This method preserves not only nucleotides but also the cell architecture for future *in situ* hybridization. The remaining sputum slurry is preserved for morphology and immunostaining in PreservCyt (Cytoc Corp). The Cytoc ThinPrep process is used to prepare slides for Pap and immunostaining.

IDENTIFICATION OF MOLECULAR MARKERS

Abnormal Methylation

Sputum tests now offer great promise to determine a molecular diagnosis of lung cancer, far in advance of clinical presentation. Although none of these molecular endpoints has been validated for clinical use, several are in clinical trials, and data from application of marker assays to archived sputum specimens look promising. Herman et al²⁰ have described a DNA-based assay that detects gene promoter regions that are hypermethylated aberrantly. The addition of methyl groups to a sequence motif (CpG islands) in the gene promoter region results in gene transcription failure, effectively an alternative to coding region mutation for loss of tumor suppressor gene function.¹⁶ Epigenetic CpG island methylation-induced transcriptional repression, at least partly, can be reversed by cell culture treatment with the demethylating agent 5-aza-2'-deoxycytidine.³⁰

Belinsky et al⁵ recently showed that hypermethylation of the CpG islands of the *p16* gene, a suppressor of the cell cycle, can be demonstrated in the sputum of patients in the early stages of non-small cell lung cancer. Palmisano et al³³ showed in specimens archived by Saccomanno that aberrant methylation of the *p16* or 0'-methylguanine-DNA methyltransferase promoters can be detected in DNA from sputum in 100% of patients with squamous cell lung carcinoma up to 3 years before clinical diagnosis. These investigators suggest that detection of *p16* CpG island hypermethylation might be useful in the prediction of individuals who might develop lung cancer. As of yet, however, no prospective studies have been done to assess the performance of the hypermethylation assay on samples from individuals at risk for developing lung cancer.

ABNORMAL PROTEIN (hnRNP A2/B1) OVEREXPRESSION

During the Johns Hopkins Lung Project (JHLP), an archive of sputum specimens and associated

clinical data linking specimens to lung cancer outcome was developed. To identify cancer-associated protein overexpression, promising clinically available antibodies plus a series of murine monoclonal antibodies raised by colleagues at the National Cancer Institute were tested. Differential display of two of these monoclonal antibodies (MoAbs 703D4, 624H12) identified biomarkers of lung cancer in archived sputum specimens 2 years before clinical detection of lung cancer.⁴⁵ For these JHLP-archived specimens with moderate or severe atypical metaplasia, these antibodies together showed a sensitivity of 91% and a specificity of 88% for the diagnosis of lung cancer within 2 years.

Results thus far indicate that MoAb 703D4 recognizes an epitope of hnRNP A2 and its splice variant, hnRNP B1.⁵² hnRNPs are members of a family of ribonuclear proteins that generally are believed to regulate the shuttling of nascent RNA transcripts between the nucleus and cytoplasm. Interactions of these molecules also are believed to regulate mRNA splicing, capping, and polyadenylation.⁸ The hnRNP A2/B1 family of antigens frequently is observed in transformed bronchial epithelium,⁵¹ and its increased expression is associated with a critical phase of fetal lung development for three mammalian systems, suggesting an oncofetal role for this protein.²⁹

MoAb 703D4 binds hnRNP A1/B1 in selected epithelial cells exfoliated in the sputum.⁵³ In all cells correctly diagnosed by immunocytochemistry, at least a proplastic morphology was recognized. Proplasia consists of minimal cytologic changes that usually are regarded as normal epithelial responses to proliferative stimuli. To ensure consistency in the selection of proplastic cells and to reduce the possibility of false-positive diagnoses, a set of morphologic criteria based on the original description of these cells by J. K. Frost were agreed on.¹³ These morphologic criteria reflect proliferative changes in nuclear morphology and a level of cytoplasmic immaturity. When such cells bind monoclonal antibody, the authors consider them sentinel cells for preclinical lung cancer.⁵³

Detection of hnRNP A2/B1 up-regulation in morphologically normal-appearing sentinel cells permits greater generalizability of results compared with the results from the earlier JHLP specimens with moderately or gravely atypical metaplastic appearance. After the first year of the Lung Cancer Early Detection Working Group trial, 13 second primary lung cancers (SPLCs) were identified.⁴⁷ The sensitivity and specificity of the hnRNP A2/B1 biomarker for later SPLC were 77% to 82% and 65% to 81%, respectively. Among the cases called positive by immunocytochemistry and image cytometry, 67% developed SPLC within 1 year. This diagnostic accuracy exceeds that commonly found in prostate specific antigen (PSA) cancer screening tests.^{27,50} Working independently, Sueoka et al⁴² recently have published confirmation of this epitope to detect preclinical lung cancer in Japan

and propose to initiate lung cancer screening in that country.⁴² Detection of hnRNP A2/B1 overexpression in sputum epithelial cells with proplastic morphology seems to be the basis of a cytotox that could initiate a strategy of preclinical lung cancer diagnosis.

QUANTITATIVE SPUTUM CYTOLOGY BASED ON HIGH-RESOLUTION, AUTOMATED IMAGE CYTOMETRY

An alternative approach to previously discussed molecular markers to improve the sensitivity and specificity of detection of early lung cancer is based on measurements of nuclear features of epithelial cells found in sputum. This method is based on the observations that, even in the absence of any diagnostic cells in the sputum, it is possible to detect subjects with early lung cancers,³¹ as was demonstrated earlier in peripheral blood smears.¹⁸ The measurements of normal-appearing cells have been demonstrated, even if extracted several centimeters away from the early lung cancers, and can reveal the presence of malignancy in the lung. This method then was used to determine if the presence of lung cancer in negative sputa from historic slides from the Mayo Clinic Study could be detected,¹² which was positive in a limited sample size.³⁵

In this approach, historic slides that were used for qualitative (conventional) sputum cytology or newly prepared slides could be used. Historic slides first must be destained from Papanicolaou and then restained with a Feulgen-Thionin stain that stains only DNA in a stoichiometric way. Several thousand of the cell nuclei then are measured by using a fully automated, high-resolution image cytometer.³² Images of cell nuclei are captured at a high spatial resolution (~ 0.1 mm² per pixel, ~500 pixels per nucleus) and photometric resolution (>200 gray levels), and more than 100 different nuclear features, such as shape and texture features, are extracted from each nucleus. Using these features, the cells automatically are classified to different cell types, and then selected cell types are used to classify the sample as coming from a subject with or without early malignancy in the lung.

A large field study involving seven institutions of Canada, the United States, Germany, France, and Japan was conducted using this approach, where an independent learning set and test set were built from more than 150 patients with early lung cancer and 300 matching high-risk negatives. The results were encouraging, achieving overall sensitivity of 55% to 65% at 85% specificity.

More recently, a commercial company (Perceptrix Medical, Vancouver, Canada), in collaboration with the British Columbia Cancer Agency, has improved this method significantly, achieving sensitivity of 70% to 80% at a specificity of 90% for early lung cancers (stage 0 and I) for central and peripheral lung cancers. It now is projected

that this approach will undergo controlled clinical trials for approval applications in the fall of 2002. If the method is validated at the indicated levels, it could be implemented rapidly into routine clinical use. It could be carried out in a cost-effective way for large-scale screening applications, followed by emerging molecular markers, low-dose spiral CT scanning, and fluorescence bronchoscopy where appropriate.

DISCUSSION

After the well-publicized report of ELCAP,¹⁹ a new wave of enthusiasm for screening, using low radiation-dose helical CT scanning (a noninvasive and rapid test), has increased. Studies are underway to evaluate the effectiveness of this approach for the early diagnosis of lung cancer. The sensitivity and specificity of widespread CT scanning will depend on the selection of patients and the geographic area of screening. In regions of high endemic granulomatous disease, such as histoplasmosis in the Mississippi valley and coccidioidomycosis in the southwest, the high prevalence of single and multiple nodules will be present, along with small lesions that ultimately will prove to be malignant. Observing indeterminate thoracic opacities by follow-up screening to determine growth is a likely index of malignancy and probably will help identify those patients who should receive a biopsy for confirmation. Widespread CT scanning, however, cannot identify small intraepithelial lesions of the central airways. Sputum cytology or alternative sputum markers of tumors, such as those previously described, will be needed to complement CT scanning. When malignancy or high stages of dysplasia are found in sputum, fiberoptic bronchoscopy will be needed to identify the central lesions. It already has been established that the advent of light-intensified fluorescent endoscopy is more sensitive in finding tiny intraepithelial lesions than white-light bronchoscopy.²⁵

In a new screening study at the Mayo Clinic in Rochester, Minnesota, smokers older than the age of 50 years have been enrolled in sputum cytology testing. In a preliminary report presented at the American Thoracic Society in San Francisco in May, 2000, Jett et al²¹ reported finding 21 cases of cancer, of which 19 were prevalence and 2 were incidence cases. Of these, spiral CT scanning detected 19 cases, and 2 were detected by sputum cytology.²¹ This study was not restricted to patients with air flow obstruction. Spirometry was normal in 7 subjects and abnormal in 14 subjects. Seventeen of the cancers were non-small cell, and 15 of the 17 were stage I or II at the time of diagnosis. The study concluded that spiral CT scanning and sputum cytology are complementary tests for lung cancer detection.

The combination of sputum tests with helical CT scanning offers great promise for detecting lung cancer far in advance of clinical presentation. Any or all of these tests could be incorporated into

the routine management of individuals at risk for developing primary or second primary lung cancer; however, several issues must be considered before these tests are ready for clinical application. Test performance characteristics must be confirmed in prospective trials. For several of these tests, those trials are currently underway. The development of a management and intervention strategy appropriate to the state at which lung cancer is diagnosed is needed. For example, noninvasive chemoprevention might be appropriate in such patients. Preliminary studies of chemopreventive agents are now underway at the National Cancer Institute. Several of these agents could be delivered by inhaler to place a maximum dose directly on the transformed epithelium. It is now time to begin to plan for clinical trials that evaluate combined diagnostic and therapeutic approaches to assess their impact on the incidence of clinical lung cancer.

SUMMARY

The specialty has the knowledge and technology to change the outcome of lung cancer. Lung cancer, diagnosed in early stages, is as curable as all other cancers. Sputum cytology is the initial step in diagnosing roentgenographically occult lung cancer. Sputum cytology is complementary to CT scanning. Sputum cytology identifies small central lesions, and CT scanning discovers peripheral tiny adenocarcinomas.

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