Concurrent Fluorescence *in Situ* Hybridization and Immunocytochemistry for the Detection of Chromosome Aberrations in Exfoliated Bronchial Epithelial Cells

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OBJECTIVE: A procedure was developed to allow concurrent detection of chromosome aberrations and identification of bronchial epithelial cells.
STUDY DESIGN: Fluorescence in situ *hybridization for chromosome 7 and immunocytochemistry for cytokeratin were performed on exfoliated bronchial epithelial cells in a sputum sample from a cancer patient.*
RESULTS: The Spectrum Orange-labeled alpha satellite probe for chromosome 7 produced red fluorescence, nuclei were counterstained with 4,6-diamidino-

Parallel use of FISH and immunocytochemistry yielded rapid identification of exfoliated bronchial epithelial cells with numerical chromosome aberrations in a sputum sample from a lung cancer patient.

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2-phenylindole (blue), and cytokeratin was visualized using a fluorescein isothiocyanate (FITC)–conjugated secondary antibody (green).
CONCLUSION: This procedure allowed the rapid identification of airway epithelial cells with numerical chromosome aberrations in this sample. Ultimately, this procedure could increase the sensitivity and specificity of sputum cytology as a laboratory diagnostic tool for the early detection of lung cancer. (Acta Cytol 1997;41: 1769–1773)

Keywords: lung neoplasms; *in situ* hybridization, fluorescence; sputum; immunocytochemistry; trisomy; cytokeratin.

Ultimately, this procedure could reduce the problems associated with the use of sputum cytology as a diagnostic and predictive tool through its use on sputum samples with ambiguous morphology.

Lung cancer is the leading cause of cancer-related deaths in the United States in both men and women.[4] The prognosis for these patients is dependent on the stage of the tumor at the time of clinical diagnosis. It has been shown that several years before clinical development of lung cancer, high-risk individuals exfoliate cells exhibiting moderate to marked atypia in their sputum.[13] However, the diagnostic sensitivity and specificity of this procedure varies due to sample preparation and cytologic interpretation based upon subjective morphologic changes. Molecular cytogenetic and immunologic techniques, such as fluorescence *in situ* hybridization (FISH) and immunocytochemistry, may improve the value of sputum cytology as a screening tool for the early detection of lung cancer.[14]

In this study, the presence of trisomy 7 was assayed by FISH. Trisomy 7 was chosen as a molecular cytogenetic marker because it is common in lung tumors[9,16] and has been detected within the tumor-free margins of some resected lung tumors as well as bronchial epithelial cells of smokers with and without lung cancer.[5,7] Therefore, trisomy 7 may serve not only as a molecular marker of lung cancer but also as a prognostic marker.

Cytokeratin staining of exfoliated bronchial epithelial cells identifies their site of origin and aids in identification of nonepithelial cells (such as macrophages and neutrophils), thus allowing rapid identification of such cells with chromosome aberrations. Cytokeratin intermediate filaments are diagnostic markers of epithelial cells, and with the exception of some undifferentiated carcinomas,[8,11] pancytokeratin antibodies can effectively label both small and non–small cell lung cancer in bronchoscopic and sputum material.[1,2,8,11]

The purpose of this study was to develop a method of detecting chromosome aberrations relevant to lung cancer in exfoliated bronchial epithelial cells in sputum using FISH and immunocytochemistry.

Materials and Methods

Sputum Sample

Five consecutive deep lung sputum samples were collected from a single patient with known primary bronchogenic adenocarcinoma. Approximately 30 mL of sputum was expectorated into a specimen container filled with Saccomanno Fixative (Shandon Lipshaw, Inc., Pittsburgh, Pennsylvania, U.S.A.).

The five samples were processed using the accepted Saccomanno method of processing and evaluating pulmonary specimens.[11,14] Two monolayer smear control slides were made from each of the five samples to assess cellularity and specimen integrity. Adequacy of a sample was determined by the presence of alveolar macrophages. Sputums were viewed as adequate if 25% of the slide contains 100 or more alveolar macrophages. Once adequacy was assessed, the five specimens were pooled into a single aliquot. Slides were prepared for FISH/immunocytochemistry by using a modified cytocentrifuge Megafunnel (Shandon Lipshaw) slide procedure for the processing of pulmonary samples.

The sputum sample was diluted by adding 150 µL of the vortexed sample to 18 mL of diluent (50% Saccomanno Fixative and 50% 0.15M NaCl). One mL of this diluted sample was transferred by pipette into a Megafunnel chamber apparatus and applied to each by centrifugation. This method of preparation resulted in a uniform single cell layer that was contained within the rectangle marked on the Megafunnel slides.

Cell Culture

Human foreskin keratinocytes (HFKs), which are diploid and express cytokeratin, were used to optimize the FISH/immunocytochemistry technique for hybridization conditions and antibody concentration. HFK cells were grown in Keratinocyte-SFM medium (Gibco BRL, Grand Island, New York, U.S.A.). In passage 4 they were fixed in Saccomanno's fixative,[12] and cytocentrifuge samples were prepared for FISH/immunocytochemistry.

FISH Hybridization

Microscope slides were denatured at 75°C in 70% formamide for five minutes according to the manufacturer's instructions (Vysis, Downers Grove, Illinois, U.S.A.). Slides were then dehydrated, successively, in 70%, 85% and 100% ethanol for one minute each. The cells were hybridized with 2 µL of SpectrumOrange-labeled (Vysis) alpha satellite probe (CEP, Vysis) for the centromere of chromosome 7 overnight in a humidified chamber at 37°C. Following hybridization, the slides were washed successively three times in 50% formamide, once in 2× saline sodium citrate (SSC) and once in 2× SSC/0.1% NP-40 (Vysis) for 10 minutes each at 45°C and allowed to air dry in the dark.

Cytokeratin Immunocytochemistry

Hybridized slides were rinsed twice, for five minutes each, in 2× Dulbecco's phosphate-buffered saline (DPBS) with 0.1% Brij (Sigma, St. Louis, Missouri, U.S.A.) and then washed for 30 minutes in the same buffer. The slides were incubated with a 1:400 dilution of antihuman pancytokeratin monoclonal antibody (Sigma, clones C-11, PCK-26, CY-90, Ks-1A3, M20, A53-B/A2) in 1× DPBS/0.2% bovine serum albumin (BSA) (Sigma) for one hour at 37°C.[17] This monoclonal antipancytokeratin antibody cocktail is broadly reactive to epitopes present in both normal and neoplastic human epithelial cells. Following incubation, the slides were washed twice for five minutes in 2× DPBS/Brij. They were then incubated with a 1:128 dilution of fluorescein isothiocyanate (FITC)–conjugated sheep antimouse IgG (Sigma) in 1× DPBS/0.2% BSA for 30 minutes at 37°C and washed twice for five minutes in 2× DPBS/Brij and once in 1× DPBS. Finally, the slides were counterstained with 10 µL of 4,6-diamidino-2-phenylindole (DAPI) (1,000 ng/mL, Vysis).

The slides were viewed with an Olympus BX60 fluorescence microscope (Tokyo, Japan) equipped with a DAPI/FITC/tetramethyl rodamine B isothiocyanate (TRITC) triple bandpass filter (Chroma Technology, Brattleboro, Vermont, U.S.A.). Use of this filter allows the simultaneous viewing of red, green and blue fluorophores.

Results

Figure 1 shows FISH for chromosome 7 in an HFK cell stained for cytokeratin. The cells have a thin rim of green fluorescing cytoplasm, two rhodamine-labeled chromosome 7 centromere signals and a blue, DAPI-counterstained nucleus. Figure 2 shows an airway epithelial cell from a lung cancer patient exhibiting trisomy 7. The bright green cytoplasm, FISH signals and DAPI-counterstained nucleus are easily discernible. Using this technique, polyploid neoplastic cells with high nuclear/cytoplasmic ratios were easily detected in the sputum sample. Oral squamous cells were identified and could be distinguished from airway epithelial cells by characteristic morphology and amount of FITC staining

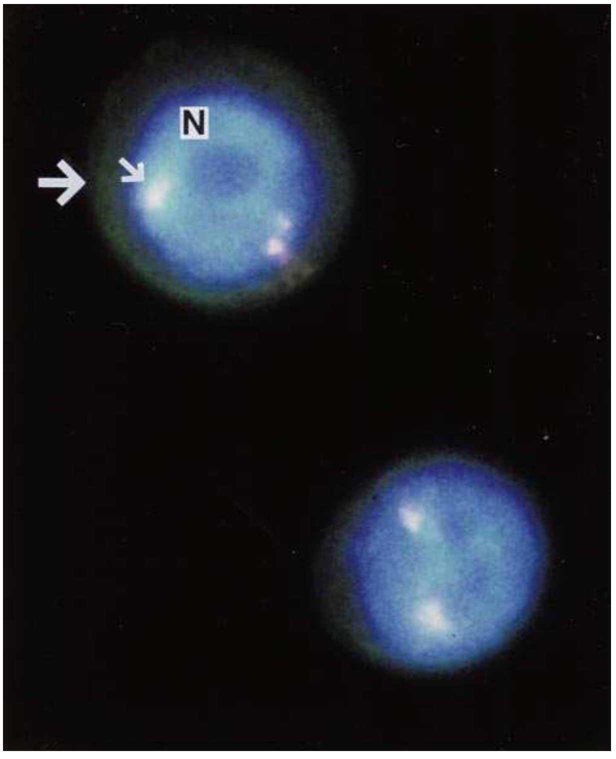

Figure 1 FISH for chromosome 7 with a SpectrumOrange-labeled alpha satellite probe (small arrow) on HFKs stained with antipancytokeratin antibodies (FITC) (large arrow), counterstained with DAPI (N) and viewed using a triple bandpass (DAPI/FITC/TRITC) filter (×150).

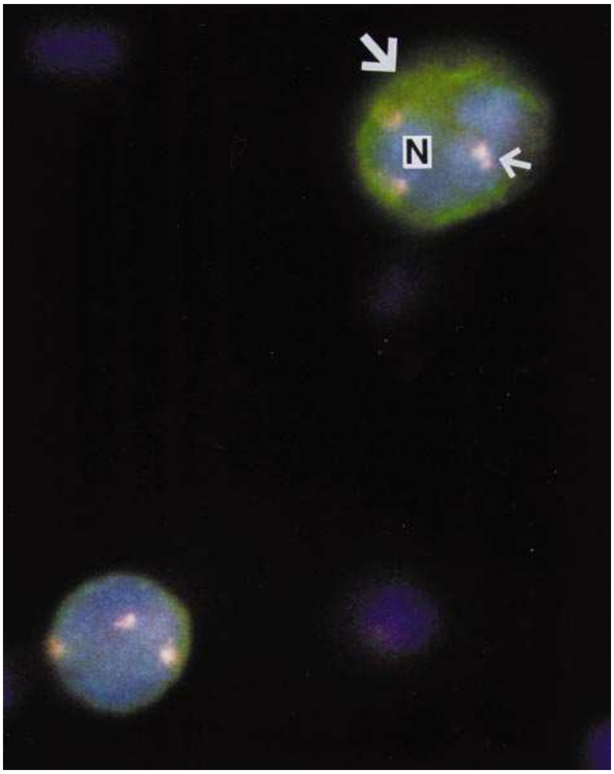

Figure 2 Exfoliated bronchial epithelial cell from the sputum of a cancer patient showing trisomy 7. Cytokeratin expression is visualized with FITC (large arrow). Chromosome 7 is detected with a SpectrumOrange-labeled alpha satellite probe (small arrow); the cell is counterstained with DAPI (N) and viewed through a triple bandpass (DAPI/FITC/TRITC) filter (×150).

in the cytoplasm (Figure 3). Neutrophils were identified by the lack of FITC staining and segmented nuclei.

Discussion

The results of this study indicate that concurrent use of FISH and immunocytochemistry on sputum samples will facilitate identification of epithelial cells with numerical chromosome aberrations. Although immunocytochemistry has been performed on sputum,[1,10,17] to our knowledge this was the first study to combine immunocytochemistry and FISH. This procedure is simple and rapid, involving hybridization with a commercially available FISH probe on day 1, posthybridization washes and FITC staining on day 2. A fluorescence microscope with a triple bandpass filter readily reveals epithelial cells containing FISH signals. In addition, using this procedure, other molecular cytogenetic and immunologic markers present in malignant cells could be detected in sputum. For example, loss of chromosome regions 9p21, 3p14, 21 and 25 and staining for cytokeratins CK 8 and 18 or tumor antigens could be assayed using this technique. Furthermore, use of a triple bandpass filter enables more than three fluorophores per cell to be simultaneously observed.

The five-year survival rate for patients whose lung cancer was detected by sputum cytology was 80%, and the resectability rate was 83%. Patients with tumors that were detected by other means had a five-year survival and resection rate of 10%.[3] However, differences in diagnosis of sputum samples between laboratories can be as high as 23%, and false positive or false negative cytology readings are not uncommon.[6] In addition, the cancer detected by sputum cytology may not be at an early stage of development even though it is not visible radiographically. Only 13–34% of sputum-positive

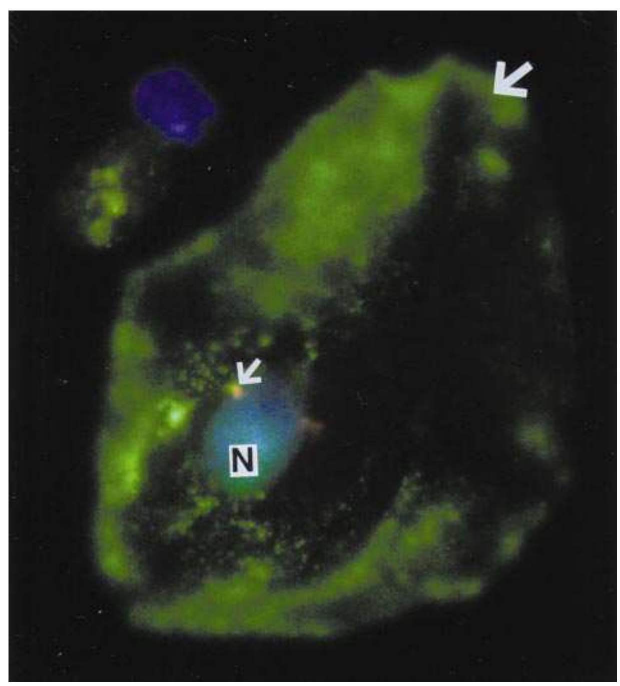

Figure 3 Diploid oral squamous cell from the sputum sample of a lung cancer patient. The cytokeratin is visualized with FITC (large arrow). Chromosome 7 centromeres are visualized following hybridization with a SpectrumOrange-labeled alpha satellite probe (small arrow); the nucleus is counterstained with DAPI (N). Note the difference in the nuclear/cytoplasmic ratio as compared to the bronchial epithelial cells (Figure 2). The cell is viewed using a triple bandpass (DAPI/FITC/TRITC) filter (×150).

but radiographically occult cancers are noninvasive.[15,18] Molecular and immunologic markers may provide a more objective means of identifying malignant or premalignant cells and may help to reduce the problems associated with sputum cytology as a diagnostic and predictive tool.

Conclusion

Parallel use of FISH and immunocytochemistry yielded rapid identification of exfoliated bronchial epithelial cells with numerical chromosome aberrations in a sputum sample from a lung cancer patient. This technique could be used with other molecular cytogenetic and immunologic markers that might be prognostic for lung cancer. Ultimately, this procedure could reduce the problems associated with the use of sputum cytology as a diagnostic and predictive tool through its use on sputum samples with ambiguous morphology.

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