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Magnetic Enrichment of Bronchial Epithelial Cells From Sputum for Lung Cancer Diagnosis

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Abstract

BACKGROUND—Sputum is an easily accessible diagnostic material for lung cancer early detection by cytologic and molecular genetic analysis of exfoliated airway epithelial cells. However, the use of sputum is limited by its cellular heterogeneity, which includes >95% macrophages and neutrophils and only about 1% bronchial epithelial cells. We propose to obtain concentrated and purified bronchial epithelial cells to improve early detection of lung cancer in sputum samples.

METHODS—Sputum was collected from patients with stage I nonsmall-cell lung cancer, cancer-free smokers, and healthy nonsmokers. Magnetic-assisted cell sorting (MACS) with anti-CD14 and anti-CD16 antibody beads were used to enrich bronchial epithelial cells by depleting macrophages and neutrophils from sputum. Fluorescence in situ hybridization (FISH) analysis for detection of *FHIT* deletion and cytology were evaluated in the enriched specimens.

RESULTS—The bronchial epithelial cells were concentrated to 40% purity from 1.1% of the starting population, yielding an average of 36-fold enrichment and at least 2.3×10^5 cells per sample. Detecting *FHIT* deletions for lung cancer diagnosis produced 58% sensitivity in the enriched sputum, whereas there was 42% sensitivity in the unenriched samples ($P = .02$). Cytologic examination of the enriched sputum resulted in 53% sensitivity, as compared with 39% sensitivity in unenriched sputum ($P = .03$). Furthermore, only 2 cytocentrifuge slides of the unenriched sputum were needed for the analyses, as compared with up to 10 cytocentrifuge slides required from the unprocessed specimens.

CONCLUSIONS—The enrichment of bronchial epithelial cells could improve the diagnostic value of sputum and the efficiency of genetic and cytologic analysis of lung cancer.

Keywords

magnetic-assisted cell sorting; bronchial epithelial cells; sputum; lung cancer; diagnosis

Nonsmall-cell lung cancer (NSCLC), mainly comprising adenocarcinomas and squamous cell carcinomas of lung, is the number 1 cancer killer in the US and worldwide.^{1,2} Given the poor prognosis associated with advanced-stage lung cancer, diagnosing the disease earlier could reduce the mortality. Chest X-ray has been used for the early detection of lung cancer; however, the sensitivity is low.³ Computerized tomographic (CT) radiologic imaging is more sensitive for diagnosing lung tumors in the peripheral lung fields than for those in the more central bronchial airways, where interpretation of CT imaging is less certain.⁴ The fluorescence bronchoscopy excels at detecting centrally occurring lung tumors.⁵ However, it is an invasive technique, and not suitable for lung cancer early detection, particularly screening populations at high risk for lung cancer. Thus, the development of sensitive and noninvasive approaches for the early diagnosis of lung cancer is clearly important to reduce mortality.

Because sputum is 1 of the most easily accessible materials and contains exfoliated airway epithelial cells from the bronchial tree, it has been considered a suitable diagnostic material for assessing carcinogenic damage in lung tumors.^{3,4} Morphologic analysis of sputum by cytology has been used for the early diagnosis of lung cancer, particularly bronchogenic squamous cell carcinoma.⁵ However, the sensitivity is very low, because the sputum cytology depends mainly on the skills required for identifying subtle morphologic abnormalities in cells. Furthermore, there is a dramatic variation in intra- and interobserver agreement in determining cancer cells because of the uncertainties of pattern recognition and classification by cytopathologists.^{3–5} In addition, because of the low percentage of bronchial epithelial cells in sputum, 10 cytocentrifuge slides are needed to have enough bronchial epithelial cells to be analyzed, making sputum cytology very labor-intensive and time-consuming.

Instead of observing morphologic characterization by cytology, molecular genetic study of sputum could identify the cells containing tumor-related genetic aberrations, which occur in microscopically normal-appearing epithelium and are specific signs of the progression of tumorigenesis.⁶ We have recently found that the combined assessment of deletions of both *HYAL2* and *FHIT* tumor suppressor genes could detect abnormal cells not only in all the cytologically positive sputum, but also in 55% cytologically negative sputum from lung cancer patients, suggesting that testing genetic aberrations in sputum could be more sensitive than cytology in identifying cancer cells.⁷ Therefore, molecular genetic studies might overcome the limitation of sputum cytology and detect genetically abnormal cells that escape cytologic examination, providing a potential diagnostic tool for early-stage lung cancer.

However, the use of sputum for molecular genetic analyses is limited by its cellular heterogeneity, which includes about 1% bronchial epithelial cells.^{8–10} The large excess of

macrophages and neutrophils that account for >95% sputum cell population could obscure detection and quantitation of neoplastic changes occurring in the bronchial epithelial cells.⁷ Therefore, enrichment of bronchial epithelial cells before the actual detection procedure is needed to improve the efficiency and accuracy of genetic and cytologic diagnosis of lung cancer in sputum samples.¹⁰

Magnetic cell sorting (MACS) is a process of immunomagnetic cell selection based on the recognition of cell-specific antibodies coupled to magnetic beads.¹¹ MACS has been developed to specifically separate rare circulating tumor cells from whole blood for predicting recurrence in patients with solid cancers.^{12–14}

The objective of the study was to obtain concentrated and purified bronchial epithelial cells from sputum to improve diagnosis of lung cancer in sputum samples. Using MACS with anti-CD14 and anti-CD16 antibody beads to specifically deplete macrophages and neutrophils, we enriched bronchial epithelial cells from sputum of stage I NSCLC patients, cancer-free heavy smokers, and healthy nonsmokers. We then analyzed the enriched sputum by using fluorescence in situ hybridization (FISH) for the detection of genetic deletion of *FHIT*.

MATERIALS AND METHODS

Patients, Sample Collection, and Preparation

Twenty-nine patients with stage I NSCLCs consisting of 15 adenocarcinomas (AC) and 14 squamous cell carcinomas (SCC) were recruited into the study. Twenty-six cancer-free heavy smokers (>40 pack-year) and 28 healthy nonsmokers were entered as controls. Written informed consent for participation was obtained through an Institutional Review Board-approved protocol.

Sputum was collected by the method described by Pizzichini et al.,¹⁵ which can maximize the yield of cells from lower respiratory airway and decrease the percentage of oral epithelial cells in the sputum. Subjects were asked to blow their nose, rinse their mouth, and swallow water to minimize contamination of squamous cells from postnasal drip and saliva. Sputum samples were then coughed in a sterile container and processed within 2 hours. To further minimize oral squamous cell contamination, opaque or dense portions that looked different from saliva under the inverted microscope were selected using blunt forceps from expectorate. The samples were processed on ice in 4 volumes of 0.1% dithiothreitol (Sigma-Aldrich, St. Louis, Mo) followed by 4 volumes of phosphate-buffered saline (PBS) (Sigma-Aldrich). The cell suspension was filtered through 45 µm nylon gauzes (BNSH Thompson, Scarborough, ON, Canada). Absolute cell numbers and cell viability were quantitated by using a hemacytometer with trypan blue. Two cytocentrifuge slides equivalent to 5000 cells per slide were prepared from aliquots of cell suspension by using a cytospin machine (Shandon, Pittsburgh, Pa) and were then stained with the Papanicolaou staining technique.^{16,17} The oral squamous cell contamination and percentage of macrophages were evaluated on the cytocentrifuge slides by pathologists. A sample was considered adequate if it had more than 2×10^7 cells, less than 4% oral squamous cells, and >50% alveolar macrophages, as described previously.^{8,9}

Magnetic Cell Sorting (MACS)

The MACS negative selection strategy was developed using anti-CD14 microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) to deplete the macrophages and anti-CD16 microbeads (Miltenyi Biotech) to remove the neutrophils from sputum samples. Up to 1×10^8 cells in 1 mL of cell suspension of sputum were incubated with the antibody microbeads on ice for 20 minutes, with occasional gentle agitation. An enrichment column (Miltenyi Biotech) with a diameter of 1.5 cm was washed with 60 mL of ddH₂O 3 times according to the manufacturer's instructions and then placed in a VarioMACS magnetic cell separator (Miltenyi Biotech). The incubated cells were fed into the column and the flow-through (eluate) was collected in a 10 mL sterile tube on ice. The eluate was centrifuged at 300g for 10 minutes. The cell pellet was then resuspended in $1 \times$ PBS as sorted samples, which contained the enriched bronchial epithelial cells.

Cytology

At least 8 cytocentrifuge slides were made from each enriched sample in single preparations by using a cytospin machine (Shandon).⁷ Four of the cytocentrifuge slides were fixed in 95% alcohol for Papanicolaou staining as described previously^{16,17} for cytologic diagnosis. The remaining cytocentrifuge slides were fixed in a 3:1 solution of methanol:glacial acetic acid for 30 minutes and then stored at -20°C for the following FISH analysis. Similarly, 20 cytocentrifuge slides were prepared from each unenriched sample by using a cytospin machine (Shandon). Ten of the slides were stained with the Papanicolaou staining technique for cytologic diagnosis. The rest of the cytocentrifuge slides were fixed in the methanol and glacial acetic acid solution for the FISH analysis. Cytologic diagnosis of the Papanicolaou-stained slides was performed by 2 senior cytopathologists using the classification of Saccomanno et al.^{16,17} Cytocentrifuge slides were screened and classified according to a 7-tiered scoring system as follows: negative, squamous metaplasia, mild dysplasia, moderate dysplasia, severe dysplasia, carcinoma, or insufficient for diagnosis. Positive cytology included severe dysplasia and carcinoma.⁴

Fluorescence In Situ Hybridization (FISH)

A specific probe for *FHIT* was prepared, labeled with green fluorescence, and tested using dual-FISH on the samples as described in our previous report.⁷ Briefly, centromeric probe for chromosomes 3 (CEP3, Vysis, Downers Grove, Ill) was labeled with red fluorescence and used as an internal control probe. The CEP3 probe was mixed with the specific probe for *FHIT* in 10 μL of LSI hybridization buffer (Vysis) and mounted on a slide. Hybridization and postwashing were done as described previously.^{7,18} The slides were examined under a microscope equipped with appropriate filter sets (Leica Microsystems, Buffalo, NY). More or less signals from the *FHIT* probe than from the CEP3 probe indicated a gain or loss of *FHIT* gene. Normal interphase cells were prepared from peripheral lymphocytes from 10 healthy subjects as described previously^{7,18} and used as controls to test the efficiency of the FISH assay and to establish cutoff values for defining abnormal specimens.

Statistical Analysis

The enrichment parameters were determined using the following calculations: Fold enrichment = percent of desired cells in enrichment/percent desired cells in start fraction. Percent purity = number of desired cells in enrichment/total number of cells in enrichment. The cutoff value to define abnormal cells with genetic aberrations of *FHIT* was calculated from normal cells and defined as the mean number of cells ± 3 SD with an abnormal *FHIT* signal pattern by FISH analysis. Associations between changes of the gene and patients' clinical diagnosis were analyzed using the Wilcoxon rank sum test for continuous variables or Fisher exact test for categorical variables. All statistical analyses were done using Statistical Analysis System software v. 6.12 (SAS Institute, Cary, NC). All *P*-values shown are 2-sided and *P* < .05 was considered statistically significant.

RESULTS

Enrichment of Bronchial Epithelial Cells

Sputum was successfully collected from 22 of 29 lung cancer patients, all of 26 heavy smokers, and 22 of 28 healthy nonsmokers. Three sputum samples from 22 lung cancer patients, 4 from heavy smokers, and 4 from healthy nonsmokers had to be excluded from the study due to excessive oral squamous cell contamination of the sputum materials (oral squamous cell accounted for >4% sputum cell population). Therefore, the sputum samples from 19 patients with stage I NSCLCs (10 AC and 9 SCC), 22 cancer-free smokers, and 18 healthy nonsmokers were finally studied in the research. The median volume of the expectorated sample was 8.0 mL, ranging from 7.6 to 11.2 mL. All the selected sputum specimens were mucoid and of lower respiratory origin as indicated by the presence of >50% macrophages and about 1% bronchial epithelial cells and less than 4% squamous cells. Furthermore, the samples had an average of 70% cell viability.

In unsorted sputum samples, bronchial epithelial cells represent $1.1\% \pm 0.2\%$ (mean $\pm$ SD) of the total sputum cells, as assessed on the cytocentrifuge slides. After MACS exclusion of macrophages and neutrophils, the average percentage of respiratory epithelial cells from 1000 cells per enriched sample was $40\% \pm 16\%$, producing an average of 43% purity in the processed samples, resulting in an average 36-fold enrichment (Table 1). Furthermore, although the absolute number of enriched bronchial epithelial cells varies in different sputum samples, the average yield of bronchial epithelial cells in the enriched specimens was $396,862 \pm 16,682$. In addition, this method for purified isolation was accomplished in <1 hour and preserved bronchial epithelial cells in a quiescent, viable state (>60% viability). Therefore, MACS enrichment could rapidly and efficiently produce high yield (more than 2.3×10^5 per sample) and purified sputum bronchial epithelial cells (an average of 43% purity).

Detection of the Genetic Changes in the Enriched Sputum Samples

Dual-color FISH was successfully performed on all the cytocentrifuge slides prepared from normal peripheral blood lymphocytes, and unenriched and enriched cells of each sputum sample (Fig. 1). However, the probes on the enriched sputum cellular samples showed bright signals with a high signal-to-noise ratio than those on the unenriched sputum cellular

samples (Fig. 2). Furthermore, because the cytocentrifuge slides prepared from the enriched samples had >40% bronchial epithelial cells, it was easy to find the cells and count fluorescent signals of the probes on enriched samples (Fig. 2A), as compared with the cytocentrifuge slides prepared from the unenriched samples that had >95% of non-epithelial cells (Fig. 2B). For example, to manually count the signals of the probes in 200 bronchial epithelial cells it took only 10 minutes on 1 slide of the enriched sample, whereas at least 40 minutes on the slide of the unenriched sample. To find 1000 bronchial epithelial cells with satisfied fluorescent signals of the probes, only 1 slide prepared from enriched sputum is needed, whereas at least 4 cytocentrifuge slides were required from the unenriched samples. Therefore, the enrichment of bronchial epithelial cells by MACS could reduce cell sample size with high purity of respiratory epithelial cells, and allow rapid genetic analysis of sputum.

Loss of *FHIT* signals was detected in 2% to 4% of normal lymphocytes from 10 healthy subjects. A sputum specimen was therefore considered to have deletions in *FHIT* if >9% of its cells had deletions in the gene. Based on this criteria, *FHIT* deletions were found in 11 of 19 (58%) and 1 of 22 (5%) cancer patients and cancer-free heavy smokers, respectively, when using enriched samples. However, *FHIT* deletion was found in 8 of 19 (42%) and 2 of 22 (9%) unenriched sputum of cancer patients and smokers, respectively. The *FHIT* deletion was not detected in any type of sputum samples obtained from healthy nonsmokers. Overall, measuring *FHIT* deletions in enriched sputum produced 58% sensitivity and 98% specificity, and 42% sensitivity and 95% specificity in unenriched sputum samples for the diagnosis of lung cancer. Therefore, the genetic analysis of the enriched sputum could increase the detection of *FHIT* deletions ($P = .02$) and potentially be useful to improve the sensitivity of lung cancer diagnosis. The *FHIT* deletions occur in the sputum of NSCLC patients with equal frequency between AC and SCC (55% vs 50%; $P = .11$). The resulting data suggest that the enrichment of bronchial epithelial cells could improve the accuracy of genetic diagnosis of lung cancer in sputum samples.

Sputum Cytology in the Enriched Samples

The cytocentrifuge slides from the enriched sputum showed that approximately half of the cells were bronchial epithelial cells with clear background compared with the cytocentrifuge slides prepared from unenriched samples where the majority of the cells were neutrophils and macrophages (Fig. 3). Using the unprocessed samples, 7 of 19 (39%) lung cancer patients were cytologically diagnosed to be positive for lung cancer. Of these 7 lung cancer patients, 5 had SCC of lung and 2 had AC of lung. Cytologic analysis of the enriched sputum not only confirmed the 7 positive cases diagnosed by using the unenriched sample, but also detected cancer cells in sputum from 3 lung cancer patients (2 with SCC and 1 with AC) that were negative when the unenriched sputum were examined. All the sputum from cancer-free smokers and healthy nonsmokers were negative by cytology. Overall, the sensitivity and specificity of cytologic examination were 53% and 100% in enriched sputum, as compared with 39% and 100% in unenriched samples, implying that the use of the enriched sputum samples would improve the sensitivity of conventional sputum cytology in the diagnosis of lung cancer ($P = .03$). Furthermore, to cytologically diagnose lung cancer in unenriched sputum, up to 10 cytocentrifuge slides were required, whereas only 2

cytocentrifuge slides are needed for the cytocentrifuge slides prepared from the enriched cells. Therefore, the data confirm that the cytologic examination of sputum is helpful for the detection of central SCC tumors arising from the larger bronchi. More important, the enrichment of bronchial epithelial cells could reduce the number of the slides examined and labor and time consumption and, thus, improve the efficiency and sensitivity of cytologic diagnosis of lung cancer in sputum.

DISCUSSION

Our study demonstrates that the MACS enrichment for bronchial epithelial cells could average 36-fold over the original sputum in 1 step, yielding at least 2.3×10^5 cells with >40% purity per sample. Furthermore, the enrichment for respiratory epithelial cells could increase the diagnostic value of sputum and efficiency of genetic and cytology analysis of lung cancer.

To improve the detection and quantitation of neoplastic changes occurring in the rare respiratory epithelial cells from sputum, centrifugation has been used as a conventional method for preparing bronchial epithelial cells from sputum. However, it is tedious and of low yield, with poor quality of separation. For example, discontinuous density gradient centrifugation only resulted in a 1.2- to 2.4-fold enrichment of bronchial epithelial cells relative to unprocessed sputum samples from patients with lung cancer carcinoma.¹⁹ Kraemer et al.¹⁰ used the AE1AE3 cytokeratin/DNA flow-assisted cell sorting (FACS) to enrich rare diploid bronchial epithelial cells from sputum, producing a 38-fold enrichment. Compared with conventional centrifugation techniques, FACS has more selection efficiency in capturing rare cells from heterogeneous mixtures in the body fluids. However, it is limited by a relatively low sorting rate ($<1 \times 10^4$ cells/s) and number of cells processed (up to 1×10^6 cells) and the need for complicated and expensive instruments and dedicated trained staff to operate the system.²⁰ Furthermore, binding of antibody to target cells might interfere with cell function and further downstream molecular genetic diagnosis.²¹

MACS has the simplicity of the design and operation of the sorting devices, allows up to 10^9 cells separated in a single process, and has been used for the enrichment of rare cells in the diagnosis of human tumors.^{11,21} For example, Bilkenroth et al.¹² applied the MACS system to enrich HEA-positive carcinoma cells in peripheral blood from renal carcinoma patients, and thus reduced the cell sample size to 1 slide for immunocytochemical analysis. Gauthier et al.¹³ used Dynabeads to enrich BerEP4-positive epithelial cancer cells in the blood of cancer patients, followed by (TRAP)-ELISA to measure telomerase activity. However, there is no previous report of using MACS to purify cells of interest in sputum for cancer diagnosis. Here we demonstrate that MACS is an effective method to enrich bronchial epithelial cells from sputum. First, the MACS had similar enrichment efficiency (36-fold enrichment and an average purity of 43%) as compared with flow cytometric (FCM), which has a 38-fold enrichment and 42% purity.¹⁰ Second, MACS is a simple and fast enrichment strategy, because it simultaneously depletes macrophages and neutrophils, while enriching exfoliated bronchial cells in a single process, decreasing the time consumption to less than 1 hour from more than 4 hours using FCM. Finally, the MACS strategy is a relatively

inexpensive and high-throughput enrichment procedure that could easily be available and accessible in many clinical settings.²¹

Our study demonstrates that the detection of *FHIT* aberrations in the enriched sputum produced higher sensitivity compared with unprocessed sputum, implying that the enrichment could improve the accuracy of genetic diagnosis of lung cancer in sputum samples. The enrichment of bronchial epithelial cells could also improve the sensitivity of conventional sputum cytology and reduce the numbers of the cytocentrifuge slides required and labor and time consumption. Furthermore, the MACS negative strategy by retaining unwanted labeled cells in column yields high numbers of bronchial epithelial cells without binding bead and antibody. The MACS does not alter the structure, function, or activity status of enriched cells, which, therefore, could also have the potential to be used for analysis of other forms of biomarkers in the early detection of lung cancer. For example, methylations and mutations of lung cancer-related genes could be examined in the enriched cells by polymerase chain reaction (PCR)-based techniques. In addition, the effective approach for capturing bronchial epithelial cells would be regularly extended to other relatively easily accessible materials, eg, bronchial washing specimens that are also typically heterogeneous, for the diagnosis of lung cancer.

Assessing *FHIT* deletion in enriched sputum could improve sensitivity in the detection of lung cancer. However, the 58% sensitivity resulting from an *FHIT* probe is not high enough to be used for lung cancer diagnosis in routine clinical practice. It has been suggested that lung cancer early detection may rely on a panel of biomarkers rather than a single 1 to predict a clinically significant cancer phenotype with acceptable accuracy.^{22,23} In an ongoing study, we are optimizing a panel of genetic probes (including the *FHIT* probe) that could be seen in enriched sputum to detect early-stage lung tumor with both higher sensitivity and specificity.

As expected, the cytologic examination of the enriched sputum samples is helpful for the detection of central SCC tumors arising from the larger bronchi. However, *FHIT* deletions occur in the sputum of NSCLC patients with equal frequency between AC and SCC. The data suggest that the genetic analysis of sputum may not only be useful for the diagnosis of central SCC cancer, but also for peripheral AC tumors. One explanation for the finding might be that sputum contains both exfoliated cells shed from central tumors themselves arising from the larger bronchi (eg, SCC) and cells coming from fields of genetically abnormal respiratory epithelium. Cytologic evaluation could only identify the exfoliated cells shed from central SCC tumors. The genetic test not only detects the cytologically abnormal cells, but also morphologically normal-appearing cells coming from fields of abnormal respiratory epithelium whose genetic aberrations might reflect the severity of the cancerization in peripheral AC tumors, because lung cancers develop from the field of pre-malignant lesions in the airway exposed to carcinogenesis. In fact, our previous study⁷ showed that there was concordance of genetic aberrations including *FHIT* deletions in matched sputum and tumor tissues from lung cancer patients, and the genetic changes in sputum might indicate the presence of the same genetic aberrations in lung tumors. This observation is also consistent with previous studies from others.^{24–26} Nevertheless, the finding needs to be validated in a large cohort study.

In summary, our current study shows that highly enriched populations of bronchial epithelial cells can be obtained from sputum by MACS, which might overcome a major limitation to conventional sputum cytology and quantitative molecular genetic analysis of sputum.

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References

1. Greenlee RT, Hill-Harmon MB, Murray T, et al. Cancer statistics, 2001. *CA Cancer J Clin.* 2001; 51:15–36. [PubMed: 11577478]
2. Parkin DM, Bray F, Ferlay J, et al. Estimating the world cancer burden: Globocan 2000. *Int J Cancer.* 2001; 94:153–156. [PubMed: 11668491]
3. Frost JK, Ball WC Jr, Levin ML, et al. Early lung cancer detection: results of the initial (prevalence) radiologic and cytologic screening in the Johns Hopkins study. *Am Rev Respir Dis.* 1984; 130:549–554. [PubMed: 6091505]
4. Byers T, Wolf HJ, Franklin WA, et al. Sputum cytologic atypia predicts incident lung cancer: defining latency and histologic specificity. *Cancer Epidemiol Biomarkers Prev.* 2008; 17:158–162. [PubMed: 18199720]
5. Hirsch FR, Franklin WA, Gazdar AF, Bunn PA JR. Early detection of lung cancer: clinical perspectives of recent advances in biology and radiology. *Clin Cancer Res.* 2001; 7:5–22. [PubMed: 11205917]
6. Belinsky SA. Gene-promoter hypermethylation as a biomarker in lung cancer. *Nat Rev Cancer.* 2004; 4:707–717. [PubMed: 15343277]
7. Li R, Todd NW, Qiu Q, et al. Genetic deletions in sputum as diagnostic markers for early detection of stage I non-small cell lung cancer. *Clin Cancer Res.* 2007; 13:482–487. [PubMed: 17255269]
8. Woltmann G, Ward RJ, Symon FA, et al. Objective quantitative analysis of eosinophils and bronchial epithelial cells in induced sputum by laser scanning cytometry. *Thorax.* 1999; 54:124–130. [PubMed: 10325916]
9. Belda J, Leigh R, Parameswaran K, et al. Induced sputum cell counts in healthy adults. *Am J Respir Crit Care Med.* 2000; 161:475–478. [PubMed: 10673188]
10. Kraemer PS, Sanchez CA, Goodman GE, et al. Flow cytometric enrichment for respiratory epithelial cells in sputum. *Cytometry A.* 2004; 60:1–7. [PubMed: 15229852]
11. Miltenyi S, Muller W, Weichel W, et al. High gradient magnetic cell separation with MACS. *Cytometry.* 1990; 11:231–238. [PubMed: 1690625]
12. Bilkenroth U, Taubert H, Riemann D, et al. Detection and enrichment of disseminated renal carcinoma cells from peripheral blood by immunomagnetic cell separation. *Int J Cancer.* 2001; 15:577–582. [PubMed: 11304694]
13. Gauthier LR, Granotier C, Soria JC, et al. Detection of circulating carcinoma cells by telomerase activity. *Br J Cancer.* 2001; 2:631–635. [PubMed: 11237383]
14. Iinuma H, Okinaga K, Adachi M, et al. Detection of tumor cells in blood using CD45 magnetic cell separation followed by nested mutant allele-specific amplification of p53 and K-ras genes in patients with colorectal cancer. *Int J Cancer.* 2000; 89:337–344. [PubMed: 10956407]
15. Pizzichini E, Pizzichini MM, Efthimiadis A, et al. Indices of airway inflammation in induced sputum: reproducibility and validity of cell and fluid-phase measurements. *Am J Respir Crit Care Med.* 1996; 154:308–317. [PubMed: 8756799]
16. Saccomanno G, Archer VE, Auerbach O, et al. Development of carcinoma of the lung as reflected in exfoliated cells. *Cancer.* 1974; 33:256–270. [PubMed: 4810100]

17. Saccomanno, G. Diagnostic Pulmonary Cytology. 2. Chicago: American Society of Clinical Pathologists Press; 1986. p. 1-211.
18. Jiang F, Caraway NP, Nebiyu Bekele B, et al. Surfactant protein A gene deletion and prognostics for patients with stage I non-small cell lung cancer. Clin Cancer Res. 2005; 11:5417–5424. [PubMed: 16061856]
19. Albright CD, Frost JK, Pressman NJ. Centrifugal separation of cells in sputum specimens from patients with undifferentiated carcinoma. Cytometry. 1986; 7:536–543. [PubMed: 3022998]
20. Davidson B, Dong HP, Holth A, et al. Flow cytometric immunophenotyping of cancer cells in effusion specimens: diagnostic and research applications. Diagn Cytopathol. 2007; 35:568–578. [PubMed: 17703449]
21. Pappas D, Wang K. Cellular separations: a review of new challenges in analytical chemistry. Anal Chim Acta. 2007; 3:26–35. [PubMed: 17904469]
22. Thunnissen FB. Sputum examination for early detection of lung cancer. J Clin Pathol. 2003; 11:805–810. [PubMed: 14600122]
23. Endoh H, Tomida S, Yatabe Y, et al. Prognostic model of pulmonary adenocarcinoma by expression profiling of 8 genes as determined by quantitative realtime reverse transcriptase polymerase chain reaction. J Clin Oncol. 2004; 22:811–819. [PubMed: 14990636]
24. Ahrendt SA, Hu Y, Buta M, et al. p53 mutations and survival in stage I non-small-cell lung cancer: results of a prospective study. J Natl Cancer Inst. 2003; 2:961–970. [PubMed: 12837832]
25. Hsu HS, Chen TP, Wen CK, et al. Multiple genetic and epigenetic biomarkers for lung cancer detection in cytologically negative sputum and a nested case-control study for risk assessment. J Pathol. 2007; 213:412–419. [PubMed: 17973238]
26. Machida EO, Brock MV, Hooker CM, et al. Hypermethylation of ASC/TMS1 is a sputum marker for late-stage lung cancer. Cancer Res. 2006; 66:6210–6218. [PubMed: 16778195]

FIGURE 1.

Fluorescence in situ hybridization (FISH) analyses of normal peripheral blood lymphocytes. Normal interphase cells show 2 green signals from the *FHIT* probe and 2 red signals from the CEP3 probe. Original magnification, $\times 400$.

FIGURE 2.

Fluorescence in situ hybridization (FISH) analyses of exfoliated cells from sputum specimens. (A) A cytocentrifuge slide prepared from enriched sputum of a lung cancer patient shows a high percentage of bronchial epithelial cells characterized by elongated nuclei stained with DAPI. FISH analysis of the sample shows hemizygous deletion of *FHIT* represented by 1 green signal of *FHIT* probe and 2 signals of the CEP3 probe in some bronchial epithelial cells. Original magnification, $\times 400$. (B) A cytocentrifuge slide prepared from unenriched sputum from the same patient shows a high percentage of nonepithelial cells. The nonepithelial cells are mainly neutrophils and macrophages, which have several round nuclei or nucleus that appear segmented and has lobes with weak signals of the probes. Original magnification, $\times 400$.

FIGURE 3.

Cytological analysis of sputum sample before and after enrichment. (A) A cytocentrifuge slide prepared from unenriched cells of 1 sputum sample shows that the majority of the cells are neutrophils and macrophages with abundant debris, whereas bronchial epithelial cells are scarce (Papanicolaou stain; original magnification, $\times 200$). (B) A cytocentrifuge slide prepared from enriched cells from the same sample shows more than half of cells were bronchial epithelial cells with clear background (Papanicolaou stain; original magnification, $\times 200$).

TABLE 1**Differential Cell Counts From Enriched Sputum**

Cell Type	Mean $\pm$ SD	Range
% Bronchial epithelial cells	40.2 $\pm$ 16.4	22.6–68.9
% Macrophages	9.7 $\pm$ 4.8	3.7–14.9
% Neutrophils	4.6 $\pm$ 1.9	1.5–7.3
% Squamous cells	39.8 $\pm$ 28.2	19.3–68.4
% Other cell types	6.2 $\pm$ 2.8	3.9–9.2

SD indicates standard deviation.

Three cytocentrifuge slides were analyzed from each enriched sample; 1000 cells were counted on each slide. Other cell types included lymphocytes and eosinophils.