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Volatile Organic Compounds in Exhaled Air from Patients with Lung Cancer

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Using a specially developed breath collection technique and computer-assisted gas chromatography/mass spectrometry (GC/MS), we have identified in the exhaled air of lung cancer patients several volatile organic compounds that appear to be associated with the disease. The GC/MS profiles of 12 samples from lung cancer patients and 17 control samples were analyzed by using general computerized statistical procedures to distinguish lung cancer patients from controls. The selected volatile compounds had sufficient diagnostic power in the GC/MS profiles to allow almost complete differentiation between the two groups in a limited patient population.

Additional Keyphrases: *gas chromatography/mass spectrometry · diagnostic screening · sample collection · data processing*

The clinical value of early detection of presymptomatic lung cancer has not yet been established. Current controlled trials of screening are aimed, in part, at resolving the issue of whether the observed increased survival of patients with such tumors is real or apparent (*1, 2*). Diagnostic techniques currently used in these experimental studies of early detection (ultraviolet-assisted fiber-optic bronchoscopy, cytology) are costly and invasive, obviating their applicability to large-scale screening efforts. Alternative methods for early detection should therefore be explored.

The presence in expired breath of certain volatile organic compounds in above-normal concentrations suggests a means for such medical diagnosis (*3*). Various analytical procedures have detected and quantified more than 200 compounds in the expired air of human subjects (*3–9*). The technology has originated from efforts to identify environmental pollutants in ambient air at concentrations of parts per billion (10^{-9}) or less (*10*). Although the metabolic pathways by which compounds present in expired air are derived are generally unknown, interest in the clinical diagnostic potential of breath analysis is evidenced by preliminary characterizations of expired air in renal failure (*11*), liver disease (*12–14*), and diabetes (*9, 15–17*).

In this study, we explored the possibility that the expired air of subjects with lung cancer might contain unique volatile organic compounds that could be used to develop noninvasive diagnostic screens. We used a computer-assisted methodology to extract characteristic volatile organic compounds from the expired breath of subjects with lung cancer and from controls, combining the use of gas chromatography/mass spectrometry (GC/MS) and multivariate statistics.

Materials and Methods

Selection of Subjects

Lung cancer patients. Samples of expired breath were collected from 14 consecutive subjects of both sexes admitted to Michael Reese Hospital and Medical Center for diagnosis and treatment.[4] The diagnosis of lung cancer was established in each case by bronchoscopic or fine-needle biopsy of pulmonary lesions. Samples of expired air were collected before chemotherapy or radiation therapy was instituted. Comprehensive records of medications taken during the four- to six-week period preceding sample collection were available. There was no consistent pattern of medication used among these subjects; two subjects took thiazide diuretics, two used propranolol, one used digitalis, and three took aspirin or acetaminophen intermittently. In two cases, the GC/MS data were technically unsatisfactory because of unacceptably high background. None of the subjects had any significant co-morbid factors such as infections or liver/renal failure. The characteristics of the 12 patients studied are summarized in Table 1.

[4] Hospitalized subjects were recruited by written informed consent. The protocols were approved by the Michael Reese Human Research Committee.

Table 1. Description of Lung Cancer Study Subjects

Age, yr	Occupation	Cigarette-smoking history, pack-years[a]	Diagnosis
48	Steel worker	25	Poorly differentiated squamous cell carcinoma, left upper lobe, bilateral mediastinal nodes and cerebral metastasis.
53	Ink factory worker	10	Large-cell carcinoma right lung, right-sided pleural effusion. Left mediastinal enlargement.
58	Truck driver	40	Squamous cell carcinoma, left upper lobe. Left hilar enlargement. Cerebral metastasis.
68	Office worker	30	Adenocarcinoma left upper lobe, upper mediastinum, right hilum.
68	Businessman	82	Adenocarcinoma left upper lobe. Left hilar enlargement, hypercalcemia.
54	Salesperson	30	Adenocarcinoma, left hilar mass, predominantly extrabronchial.
54	Security guard	40	Adenocarcinoma, right upper lobe. Rib metastasis.
56	—	60	Squamous cell carcinoma, left upper lobe. Left pulmonary artery invasion.
78	—	50	Adenocarcinoma left lung. Left-sided pleural effusion.
55	Printer	74	Undifferentiated carcinoma, cavitary (with *Aspergillus hifae* in cavity).
64	—	50	Poorly differentiated adenocarcinoma of tracheal bifurcation.
78	Housewife[b]	58	Squamous cell carcinoma, left upper lobe. Left hilar metastasis.

[a] Years of cigarette consumption at rate of one pack per day.
[b] All other subjects were male.

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Control subjects. We also studied 17 samples of expired air from a control group of nine normal subjects, ages 25 to 70 years, including one woman. Two of the men were heavy cigarette smokers. None of the subjects had a history or physical evidence of pulmonary or systemic disease. Most of these control subjects were hospital personnel.

Sampling and Analysis

Sample collection. A specially designed system was used to collect samples of expired breath on 60/80 mesh Tenax GC (Alltech Associates, Arlington Heights, IL) sorbent cartridges for later analysis of the organic vapors by GC/MS. The system, described elsewhere (*5, 6*), was designed to isolate the subject from environmental contaminants temporarily and to provide normal breathing conditions, as shown in Figure 1.

In brief, a sterilized Rudolph breathing valve (H. Rudolph, Inc., Kansas City, MO) was connected to the breathing manifold and a spring-loaded clamp attached to the person's nose. For 5 min the subject inhaled purified air from the reservoir through the mouthpiece attached to the valve and exhaled through the valve into the atmosphere. This served to minimize the presence of any environmental contaminants in the lungs inhaled during ordinary exposure. The Rudolph valve was then connected to the heated expired-air delivery tube, and the subject was asked to fill the 40-L sampling bag. After each of two such fillings, the bag was emptied by applying external pressure to it with air from the spirometer. The third filling of the bag to capacity constituted the expired air used as the sample. During this filling, the spirometer measured the subject's ventilatory rate (tidal volume and frequency).

After collecting the sample, we attached a Tenax GC cartridge to the outlet valve of the sampling bag and used a pump to draw the expired air through the cartridge, measuring the total volume of air transferred (about 20 L) with a wet test meter (Sargent-Welch Scientific Company, Skokie, IL).

GC/MS analysis. The Tenax cartridges were analyzed for volatile organic compounds by a thermal desorption GC/MS procedure that has been described in detail elsewhere (*10, 18*). Desorption was performed by heating the cartridge in an inert gas stream and collecting the volatiles in a trap cooled with liquid nitrogen. Heating the trap rapidly while flushing it with carrier gas transferred the components onto a high-resolution capillary GC column, which was coupled to a mass spectrometer and data system for cyclic scan analysis.

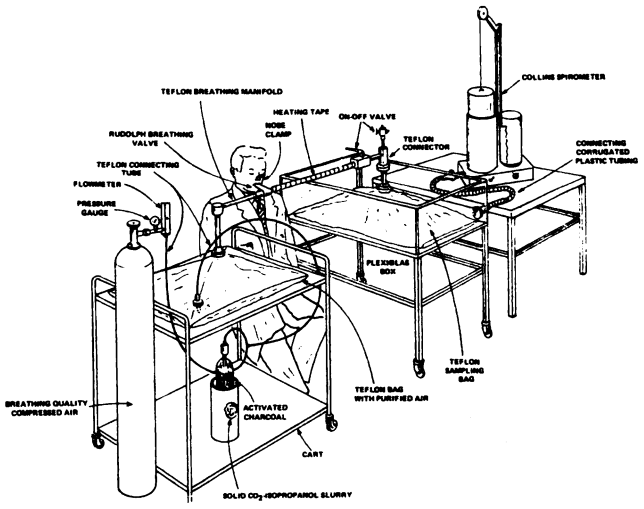

Fig. 1. System for collecting expired air samples

To improve the quality of the mass spectral data and permit comparisons between GC/MS data sets, we applied an efficient spectrum-enhancement algorithm (*19*) to the raw data. This program automatically locates and extracts components from the data to produce a set of clean spectra, i.e., free of background contributions and contaminating compounds. Once a clean spectrum has been defined by the program, its area is calculated and used to evaluate the concentration of a component in the sample by comparing the peak area of that component with the peak area of the internal standard. The standard, perfluorotoluene, was added to each Tenax GC cartridge just before analysis.

To match peaks and generate composite GC/MS profiles, a second program subjected each unknown spectrum to retention-time scaling, then passed it to an historical library matching program (*20*). Retention-time scaling is achieved by converting the raw chromatographic retention data (spectrum numbers) into a set of standardized retention indices relative to a set of marker peaks that occur throughout the data base and are easily located. The matching program defines a time window, then compares each unknown spectrum with each reference spectrum in the window of the library by performing a relative retention index (RRI) match and a spectrum match. Matching effectiveness is expressed in terms of a score that is a measure of spectral similarity and RRI proximity. (The historical library is initialized by designating one of the profiles of interest as the library.) When a satisfactory match occurs, the peak is added to the historical library and the relative concentration and RRI data for the entry are updated.

In this way, we prepared composites of individual GC/MS profiles and established statistics on the frequency of occurrence and reproducibility of the average peak intensities in the two sample populations of interest. We then applied various statistical tests to determine differences between the respective composite GC/MS profiles.

Statistical Methods

We used three general statistical procedures to reduce the dimensionality of the data and to establish relationships within the data from each group objectively. As a first screening for each of the 297 compounds in the lung cancer and control samples, we used the Fisher exact test to examine the null hypothesis of no association between lung cancer and the frequency of occurrence for each compound separately. A type I error rate of $p < .05$ indicated significance in this initial screen.

For the 57 breath compounds that were present in at least 50% of the patients and 35% of the controls, both *t*-statistics and Mann–Whitney *U* statistics were computed to test the null hypothesis of no difference in the concentration of each compound between patients and controls. In light of these multiple comparisons, we allowed an overall false-positive rate of 5% (i.e., $p < .05$) for individual compounds that have a probability of $p < .001$ (based on the Bonferoni inequality). Thus, probability values $< .001$ are significant in and of themselves, despite the large number of comparisons performed.

These statistical procedures allowed us to select 22 peaks as a starting point for developing a discriminant between GC/MS profiles from lung cancer patients and normal persons. We constructed a discriminant function to yield a linear combination of compounds that maximally differentiated patients from controls, then tested the accuracy of the estimated function both statistically and by examining the concordance between the observed and estimated classification. We considered significant a classification accuracy of greater than 80%.

Ideally, validation of the result should be based on the classification of an independent data set. However, because no unknown samples were available for prediction, we used the "jackknife" validation procedure (*24*) in an effort to minimize this bias. In this approach, a classifier is constructed on the basis of 28 (of the 29 known) samples and the remaining sample is classified. This sample is then returned to the sample set, another sample is removed, a new classifier is developed for this set, and the deleted sample is classified. This procedure continues until all samples in the set have been deleted and classified once.

Results

The GC/MS profiles of expired air from lung cancer patients and controls were complex, generally containing about 150 peaks each. Figure 2 shows typical GC/MS profiles (plots of total ion current vs spectrum number or retention time) obtained for a lung cancer patient and a control subject; obviously, visual inspection alone is unlikely to reveal subtle differences in the relative intensities of peaks in the data sets.

A list of the 49 peaks identified by RRI for which there were statistically significant differences in percent peak occurrence and (or) concentration, based on the statistical screening procedures described above, is available on request from the authors or from the Editorial Office of this journal.

In this group of lung cancer patients, four peaks occurred in more than half of the samples but were completely absent from the controls. If this observation is confirmed in a large population, the presence of these four peaks might alone provide sufficient diagnostic power in the GC/MS profiles to allow unambiguous differentiation between the lung cancer and control groups. However, the fact that the peaks did not occur in all the lung cancer patients indicates that a diagnostic approach based exclusively on their presence would probably have a false-negative rate that would be unacceptable for screening purposes.

To obviate this possibility, we selected the 22 peaks showing the largest differences in peak percent occurrence and (or) concentration (Table 2) and used them to develop a linear discriminant function for classifying GC/MS profiles from either lung cancer or control subjects. Mass spectral analysis showed that the candidate substances included 16 oxygen-containing compounds and four sulfur-containing compounds. With the linear discriminant function we developed, all 29 samples in the data set (17 from controls and 12 from lung cancer patients) were correctly classified; moreover, results were identical for both raw and jackknifed values.

Although we initially entered 22 variables into the analysis, only seven of the peaks were needed for full discrimination; their RRIs were 611, 653, 690, 739, 794, 841, and 1248. However, three of these peaks occurred in fewer than 35% in one of the two sample groups. Because these low frequencies could have biased the result, we removed from the original list of 22 peaks all variables with occurrences below 50%. Consequently, 10 peaks (RRIs 524, 611, 680, 739, 789, 841, 1280, 1299, 1374, and 1499) were used to define a new discriminant function. This function accurately classified 93% of the samples (27/29) with use of only three peaks (RRIs 611, 680, and 841). Thus, despite the restriction on the peaks and their frequencies of occurrence, the linear discriminant analysis procedure was able to distinguish between the lung cancer and control samples highly accurately, based on significant differences in the concentrations of

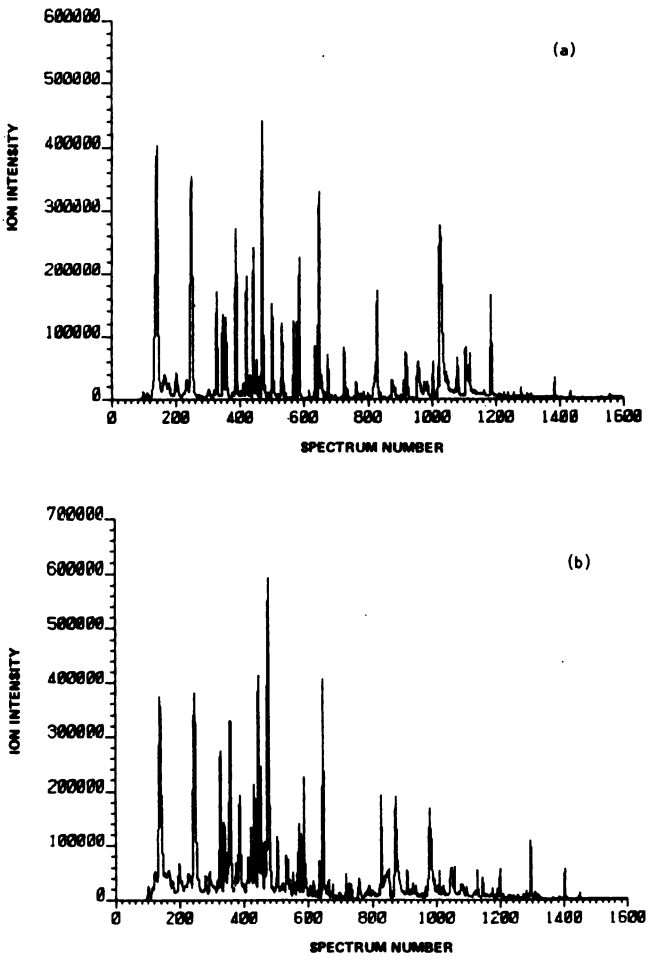

Fig. 2. Plots of total ion current vs spectrum number for GC/MS analysis of expired air samples from (*a*) lung cancer patient and (*b*) normal (smoker) subject

Table 2. Potentially Significant Diagnostic Peaks Based on Fisher Exact and Group Comparison Tests

Relative retention index[a]	Peak presence/absence: % Occurrence: Lung cancer	Peak presence/absence: % Occurrence: Control	Peak presence/absence: *p* value[b]	Group comparison, *p* value[c]: Mann–Whitney	Group comparison, *p* value[c]: *t*-test
524	91.7	82.4		0.0010	0.056
611	100.0	100.0		0.0013	0.004
653	75.0	35.3	0.0407	0.0176	0.038
659	50.0	0.0	0.0019		
680	100.0	100.0		0.0213	0.001
690	8.3	64.7	0.0030		
723	0.0	58.8	0.0010		
725	91.7	35.3	0.0030	0.0067	0.002
739	100.0	88.2		0.0002	0.000
764	0.0	58.8	0.0010		
779	100.0	35.3	0.0004	0.0242	0.000
789	100.0	94.1		0.0300	0.002
794	33.3	82.4	0.0106		
829	66.7	17.6	0.0106		
841	100.0	82.4		0.0003	0.000
1095	50.0	0.0	0.0019		
1248	75.0	0.0	0.0001		
1280	91.7	76.5		0.0039	0.022
1299	100.0	100.0		0.0040	0.003
1374	91.7	58.8		0.0019	0.010
1499	100.0	94.1		0.0026	0.001
1512	66.7	41.2		0.0001	0.000

[a] See text for definition. [b] Fisher Exact Probability Test. [c] Group comparisons calculated only for those cases in which occurrence exceeded 50% for lung cancer group and 35% for control group.

the compounds between the two groups.

The statistical analyses described here show that the development of a discriminant that separates the lung cancer profiles from the normal profiles does not require a knowledge of the chemical identity of the selected peaks. Nevertheless, the availability of the mass spectral data for each sample allows us readily to identify these as well as other peaks that may have clinical diagnostic value. In the above analysis, for example, the three peaks that gave a classification accuracy of 93% correspond to acetone, methyl ethyl ketone, and *n*-propanol.

Discussion

The results of this study suggest that unique volatile compounds of potential diagnostic usefulness are present in expired air of patients with lung cancer. Data from a small sample of subjects diagnosed late in their disease were contrasted with those from a small control population. We are aware that experimental variables such as donor gender, donor age, and GC column batch can confound the chemical information and affect the classification process (*21*), and that misleading results are possible if the ratio of the number of samples to the number of variables (peaks) is less than about three (*21, 22*). In addition, other confounding factors may have played an uncertain role. We doubt that the hospital environment was a significant factor, because most of the controls were hospital personnel. Similarly, there was no consistent pattern of medication usage that could have accounted for differences in expired air composition. However, it is possible that systematic differences in diet existed between controls and patients with lung cancer, and these may have contributed to some of the discriminant power.

Nonetheless, we have obtained sufficient relevant information to justify the need for a large-scale, systematic study in which these and other confounding factors are taken into account by use of statistical methods. The analytical and statistical approaches described here clearly provide a powerful means of selecting diagnostically significant compounds in expired breath and establishing their diagnostic power and limitations for the classification of GC/MS profiles. Applying these procedures to a larger sample population should help confirm, refute, or expand the significance of the compounds selected in this preliminary study and establish diagnostic criteria for general use. The classification function finally developed could then be used in a dedicated analytical system to classify unknown samples rapidly and reliably.

Most earlier work in diagnostic applications of expired gas analysis has contributed information of minor clinical value (*3*). However, the approach has generally been to study one or a few constituents, selected on the basis of their presumed relevance. Our approach contrasts with earlier studies in that we search concurrently for multiple volatile compounds, using state-of-the-art computer-assisted analytical technology and taking advantage of the potential diagnostic value of simultaneous changes in the concentrations of several constituents. A disadvantage of the present approach is its cost and the reliance on sophisticated technology. Our results suggest, however, that the presence of a discrete number of diagnostic compounds may suffice for identifying subjects with lung cancer. If this is confirmed by a larger study, perhaps a device based on nothing more than a high-resolution gas-chromatograph equipped with compound-specific detectors could be developed as an effective diagnostic tool. Such a device could be simple enough for potential use in a physician's consulting room as part of a routine pulmonary test.

Even if the discriminant function requires the assessment of more than a few compounds, there is still potential for simplification. Technology for such measurements is already available with the advent of the tandem mass spectrometry (MS/MS) technique. This technique, coupled with direct-sampling atmospheric pressure ionization, is capable of real-time (i.e., instantaneous) detection and identification of extremely low concentrations (parts per trillion, 10^{-12}) of compounds in air (*23*). Although these systems are still relatively expensive, the technology lends itself to miniaturization and a significant reduction in cost, once the performance requirements and mode of operation have been clearly defined.

The incidence of lung cancer continues to increase. Problems with early diagnosis and treatment still represent major clinical and epidemiological challenges. The data obtained here indicate that the analysis of expired air is feasible and could provide a noninvasive diagnostic method for use in mass screening at a relatively low cost. Further studies to develop and refine this approach are justified.

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