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New screening method for lung cancer by detecting volatile organic compounds in breath

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Abstract Lung cancer is a frequent cause of cancer-related deaths in the world. There is no valid screening process and this limits its detection to the late stages, with consequently high mortality rates. Volatile organic compounds (VOC) are chemical compounds (mainly the products of cell catabolism) found as gases in the human breath. Different methods have been developed to analyse VOCs and to compare them in healthy subjects and lung cancer patients. In this review, we summarise the different techniques used to analyse VOC. Many reports have been published with promising results similar to those achieved with accepted screening methods such as mammography. These methods show good perspectives on lung cancer screening.

Key words Volatile organic compounds • Lung cancer screening • Gas chromatography–mass spectrometry • Electronic nose • Breath analysis

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Lung cancer is the primary cause of cancer-related deaths throughout the world. From the men and women diagnosed today with lung cancer, only 14% will survive five years later. However, if lung cancer is detected promptly, the five-year survival can rise from 1% for stage IV to 67% for stage IA. For this reason, several methods are being investigated for the early detection of the disease, to thereby increase the chances of survival.

The first trials of lung cancer screening began in the 1970s, with chest radiography with or without sputum testing, but these failed to produce a reduction in lung cancer mortality, probably due to the low sensitivity of the technique [1, 2]. Recent studies performed by the US Preventive Service Task Force and the International Early Lung Cancer Action Program, using spiral computed tomography (CT), demonstrated significantly earlier diagnoses than the usual in current clinical practice [3, 4]. There is still debate about whether this technique will produce a significant decrease in the mortality rate. The US National Lung Screening Trial was begun in 2004 to address this question, and the results are expected to be ready by 2012 [5]. Another issue regarding screening with CT is its cost effectiveness. In 2003, a modelling analysis by Mahadevia et al. estimated the cost per quality-life year saved to be $US116,000 for the cohort at highest risk [6]. There is no doubt that a good screening test must be inexpensive to be applicable to all high-risk populations, and must have a very high negative predictive value (NPV).

In 1985, Gordon et al. applied a method for the microanalysis of breath to detect volatile organic compounds (VOCs) in exhaled air from patients with lung cancer [7] and later O'Neill et al. [8] determined more exactly the specific compounds found in the breath of lung cancer patients, which more accurately defined the usefulness of this technique. VOCs are emitted as gases from certain solids or liquids. A sample of human breath contains about 200 different VOCs, most of them

at picomolar (10–12 mol/l) concentrations [9]. Studies by O'Neill et al. [8] identified the chemical compositions of VOCs in lung cancer patients. Most of them were alkanes and methylated alkanes. Recent studies of oxidative stress and the generation of VOCs might offer an explanation for this association.

Generation of free radicals by reactive oxygen species (ROS) [10] is involved in cancer initiation. An increased production of ROS has been directly linked to protein, DNA and lipid oxidation. Changes induced in DNA bases may be carcinogenic by themselves, and lipid peroxidation of the polyunsaturated fatty acids in cell membranes generates alkanes, such as ethane, pentane and methyl alkanes, which are excreted in the breath [11, 12]. These compounds provide a rational explanation of the empirically observed changes in the VOC patterns of lung cancer patients.

Analysis of VOCs

Capture of the sample

In the first place a breath sample is collected from the patient and concentrated, because the concentrations of VOCs are very low. After, the patient is asked to breathe through a plastic tube connected to a sterilised valve and a Teflon sampling bag with sorbent cartridges to collect VOCs for later analysis. The system is designed to isolate the subject from environmental contaminants using a clamp attached to the person's nose, and the patients inhale purified air from a reservoir for 5 min. The system has been improved significantly, with minimisation of the dimensions of the devices and the development of better ways to concentrate breath samples before analysis.

Several methods have been used for this purpose, such as chemical interaction, adsorptive binding, cold trapping and supercritical fluid extraction, but the most successful in this field are solid-phase microextraction (SPME) and the recently developed multi-bed sorption trap. SPME was developed by Pawlhiszyn in late 1989 as a new preconcentration technology, in which a fused coated silica fibre is used as the stationary phase. The fibre coating removes the compounds from the sample by absorption. This method has been used by many researchers to preconcentrate breath samples before their analysis, with a minimum sensitivity of 10^{-2} ng/ml, sensitive enough to analyse alkanes and aromatic hydrocarbons in the human breath at concentrations ranging from parts per trillion (ppt) to parts per billion (ppb) [13–15]. The multi-bed sorption trap consists, in brief, of a metal tube packed with four discrete sorption beds. Three of the beds use different grades of graphitised carbon, and the fourth consists of carbon molecular sieves. Each bed contains about 2.2 mg of sorbent, and they are separated by plugs of glass wool. The bed ensemble is retained in the trap tube by plugs of stainless steel mesh. A recent study with the multi-bed sorption trap attached to a two-dimensional gas chromatograph detected substances in the ppt concentration range [16, 17].

Methods of analysis of VOCs

The most accurate, and therefore most widely used, is GC/MS. This method combines the features of gas–liquid chromatography and mass spectrometry to identify the different substances within a test sample [9]. However, a new method for detecting volatile substances is starting to demonstrate considerable utility, the electronic nose (Enose). The Enose detects chemical vapours, but does not perform chemical separation or identification of their components. Instead, the mixture of molecules in the gas is recorded as a pattern, and statistical pattern recognition analysis is used to determine the differences between different samples (i.e., lung-cancer breath *vs.* non-lung-cancer breath) [18, 19]. The Enose is composed of a chemical sensor system (hardware) and pattern recognition software. Using this method, we can "teach" the Enose to distinguish different patterns to be determined in the future.

As shown in Table 1, few studies have been undertaken in this field, but they show similar results. The first trial was performed by Gordon et al. [7] in 1985 with samples from 12 lung cancer patients and 17 healthy controls. Samples of expired air were collected during a 5-min inhalation of purified air and subjected to subsequent analysis with GC/MS. The authors did not analyse the exact compositions of the VOCs obtained, but simply showed the results as peaks identified by their RRI. There were statistically significant differences in the percentage peak occurrences and/or concentrations of VOCs. They selected 22 peaks that showed the greatest differences in percentage occurrence and/or concentration and used them to develop a linear discriminant function to classify the GC/MS profiles of patients with lung cancer and those of healthy controls. All samples were correctly classified with those 22 peaks.

Later on, Gordon et al. [7] then developed another discriminant function using just 10 peaks, which accurately classified 93% of the samples. They concluded that, with this method, the breath samples of healthy subjects could be distinguished from those of subjects with lung cancer. The development of a discriminant statistical analysis that distinguishes lung cancer patients from healthy subjects should also be able to identify the exact chemical identities of the selected peaks.

In 2003, Phillips et al. [20] published a study in which breath samples were collected for VOC analysis

Table 1 Publications regarding VOCs in the breath of lung cancer patients

Author	Year	Analysis method	Patients	Results
Gordon et al. [7]	1985	GC/MS	LC: 12 HC: 17	10 peaks in the RRI with 93% accuracy
O'Neill et al. [8]	1988	GC/MS	LC: 8	28 substances in >90% samples
Phillips et al. [9]	1999	GC/MS	LC: 60 HC: 48	22 VOCs classified, 81.5% correctly
Di Natale et al. [21]	2003	Enose	LC: 35 HC: 18 LC-ST: 9	94% correctly classified
Phillips et al. [20]	2003	GC/MS	LC: 87 HC: 132	9 VOCs: Sensitivity: 89.6% Specificity: 82.9%
Machado et al. [22]	2005	Enose	LC: 14 AAT: 19 CBD: 6 HC: 20	Correctly 330 of 338 samples: 85% accuracy Sensitivity: 71% Specificity: 91.9%

SPME/GC, solid-phase microextraction/gas chromatography; *LC*, lung cancer; *HC*, healthy controls; *LC-ST*, lung cancer after surgical therapy; *AAT*, α ±1-antitrypsin deficiency; *CBD*, chronic pulmonary beryllium disease

from 178 patients who had undergone bronchoscopy and from 41 healthy volunteers. Eighty-seven of them had lung cancer (15 metastatic; 67 pulmonary; five undetermined). This group identified 80 different alkanes and monomethylated alkanes that had been either synthesised or catabolised by at least one subject, by calculating its alveolar gradient. Nine of these VOCs were identified as the best markers of the disease, and their combination yielded a sensitivity of 89.6% and a specificity of 82.9% (when a 0.5 probability of disease was used as the dividing point between a positive and a negative breath test). Tobacco smoking did not significantly reduce the accuracy of the breath test; nor did the histological type or TNM stage of the cancer.

In the same year (2003), the first results of an analysis of VOCs in lung cancer patients using the Enose were published [21]. This study collected 60 individuals: 35 of them affected by lung cancer, nine after surgical treatment and 18 healthy controls. In this analysis, 94% were correctly classified. In 2005, Machado et al. [22] published the next study using the Enose. Individuals with lung cancer were shown to have a different exhaled breath profile, and a training set was used to create the model to be validated.

In the second (validation) phase, patients with lung cancer, healthy controls and control subjects with other lung diseases were evaluated in a cross-sectional blinded study. The Enose correctly identified 330 of 338 independently analysed exhaled breath samples, with an overall accuracy of 85% (95% confidence interval, 81.1–88.2%), a sensitivity of 71.4% and a specificity of 91.9%. In the group studied, which had an 18% prevalence of lung cancer, the positive predictive value (PPV) was 66.6% and the NPV was 93.4%.

Finally, this year Phillips et al. [23] reported a study using 193 subjects with primary lung cancer and 211 controls with negative chest CT. A fuzzy logic model of breath biomarkers (16 VOC) was constructed in the training and then tested in subjects in the prediction set by generating the scores for lung cancer. Mean typical scores employing a 16 VOC model were significantly higher in lung cancer patients than in the control group. This model predicted primary lung cancer with 84.6% sensitivity, 80% specificity and 0.88 area under curve (AUC) of the receiver operating characteristic (ROC). Predictions with the fuzzy logic were considered consistently superior to multilinear analysis.

Conclusions

The analysis of human breath can provide important diagnostic information. Several compounds have been studied as biomarkers, besides alkanes and methyl alkanes. Most of them are the products of cellular oxidative stress. Hydrogen peroxide levels are considerably higher in smokers and patients with chronic obstructive pulmonary disease (COPD). They also decrease in patients with asthma receiving antiinflammatory therapy. Nitric oxide (NO) concentrations are elevated in the exhaled air of patients with asthma, and it has been suggested to contribute to airway oedema and inflammation. NO can produce S-nitrosothiols, metabolites with bronchodilating effects, and levels of these are increased in the exhaled breath of smokers and patients with asthma or cystic fibrosis. Other end-products of NO metabolism, such as NO_2 and nitrate (NO_3), vary in their concentrations in exhaled air during pulmonary inflammatory diseases [24]. The end-products of lipid peroxidation are useful markers of inflammation. Increased levels of 8-isoprostane have been found in patients with asthma,

and also in the blood of many other nonpulmonary diseases associated with oxidative stress. 4-Hydroxy-2-nonenal has been suggested to play a role in the pathogenesis of COPD, because it correlates inversely with forced expiratory volume in 1 s (FEV_1). Malondialdehyde is found at high concentrations during the exacerbation of asthma in children. These two findings are similar to the determination of hydrocarbons and thiobarbituric acid reactive substances in the breath of patients with asthma or COPD [24].

Recently, Gessner et al. [25] demonstrated that DNA could also be obtained from exhaled breath condensate. They evaluated 11 patients with NSCLC and 10 healthy subjects, amplifying their DNA by polymerase chain reaction (PCR) to detect p53 mutations. They found a mutation in four (36.4%) of the lung cancer patients, and in none of the healthy subjects. This is a promising technique but its value in detecting somatic mutations in exhaled breath to detect lung cancer is yet to be demonstrated. Another study, by Carpagnano et al. [26], demonstrated the presence of microsatellite changes on chromosome 3p, detected in the exhaled breath of lung cancer patients, which strongly correlated with tobacco consumption. Although none of these markers is yet used to screen for lung cancer, they indicate the variety of information provided by exhaled breath condensate. The most reliable method so far developed is the detection of VOCs, and this has been the most intensively studied technique and it is a promising field. Low-dose CT is probably the most studied generalised technique and has already demonstrated its value, detecting 70–90% of lung cancers in stage 1 (15–20% is the usual rate for this stage) (36), but it presents several disadvantages. First, there is a high rate of noncalcified nodules, between 17% and 51%, with subsequent morbidity of follow-up scans and/or biopsies, or the resection of benign noncalcified nodules. Second, overdiagnosis can be a problem, which is, in brief, an increase in the number of diagnoses with no corresponding increase in survival. In the Mayo group screening, Fontana et al. [27] found no difference in lung cancer mortality between the screened group and the control group, but identified 29% more cancers in the first group, with no impact on the lung cancer death rate. The third disadvantage is the risk of cancer from X-rays, which are the cause of 0.6–3.2% of all cancers in developed countries. Pastorino et al. [28] investigated the efficacy of repeated yearly low-dose CT and the selective use of positron emission tomography (PET) on noncalcified nodules of >6 mm in 1035 subjects over a period of five years. This approach was useful in reducing the need for fine-needle aspiration biopsies for differential diagnoses, thus reducing the invasive techniques used. Other possibilities under study for use in lung cancer screening include light-induced fluorescence endoscopy, which is invasive and would therefore be inappropriate for screening, and sputum immunocytology, which promises much greater sensitivity than conventional sputum cytology. Sputum immunocytology involves the application of PCR to sputum cells to detect DNA changes as biomolecular markers of lung cancer [28]. Another question is how to combine these methods to achieve the best sensitivity in the detection of early lung cancer. A rational sequence would first include breath analysis, then CT, and thirdly PET. Each technique should be considered in terms of its pre-test probability to obtain the best results. A good screening test must produce few false negative results without producing too many false positive results. That is, it should display a very high NPV and a reasonable PPV.

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