




PAPER

A volatile biomarker in breath predicts lung cancer and pulmonary nodules

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Abstract

Background: previous studies have reported volatile organic compounds (VOCs) in the breath as apparent biomarkers of lung cancer. We tested the hypothesis that a robust breath VOC biomarker of lung cancer should also predict pulmonary nodules in chest CT images. **Methods: Biomarker discovery study (unblinded):** 301 subjects were screened for lung cancer with low dose chest CT (LDCT), and donated duplicate samples of alveolar breath for analysis with gas chromatography mass spectrometry (GC MS). Monte Carlo analysis of breath chromatograms revealed a mass ion as a biomarker that identified biopsy-proven lung cancer as well as suspicious pulmonary nodules on LDCT. The biomarker was termed *Mass Abnormalities in Gaseous Ions with Imaging Correlates (MAGIIC)*. The chemical structure of MAGIIC was tentatively identified from the NIST library of mass spectra; the best-fit compounds included C4 and C5 alkane derivatives that were consistent with metabolic products of oxidative stress. **Blinded validation of MAGIIC:** the abundance of the MAGIIC biomarker was determined in a different group of 161 subjects undergoing screening with LDCT. They donated duplicate alveolar breath VOC samples that were analyzed at two independent laboratories. The study was blinded and monitored with Good Clinical Practice. The abundance of MAGIIC in breath predicted biopsy-proven lung cancer with 84% accuracy, sensitivity = 75.4% and specificity = 85.0%. MAGIIC also predicted pulmonary nodules in LDCT with 80.5% accuracy, sensitivity = 80.1% and specificity = 75.0%. Breath MAGIIC abundance was not significantly affected by tobacco smoking history. **Conclusions:** in a blinded study, breath VOC MAGIIC accurately predicted lung cancer confirmed on a tissue biopsy, as well as suspicious pulmonary nodules observed on LDCT. MAGIIC may have been a product of oxidative stress and it could potentially be employed as an ancillary to LDCT to predict the likelihood that a pulmonary nodule is malignant.

The modern era of breath microanalysis commenced in 1971, when Linus Pauling reported that human breath contains large numbers of volatile organic compounds (VOCs) in low concentrations [1]. During the 1980s, researchers discovered apparently unique signals in the breath VOCs of patients with lung cancer, sparking hopes that a simple breath test could detect the disease in its early stages [2, 3]. However, this goal has been elusive; after nearly four decades of research, no breath biomarker of lung cancer has yet been adopted in clinical practice.

There is evidence from several published studies that patients with lung cancer have abnormal VOCs in their breath. This has been confirmed with different analytical methods including gas chromatography mass spectrometry (GC MS), colorimetric assays and electronic noses [4–6]. However, no consensus has yet emerged regarding the chemical identity of breath biomarkers of lung cancer: researchers have reported several different candidate compounds as diverse as *o*-toluidine [3], formaldehyde [7], alkane and benzene derivatives [5] and isopropyl alcohol [8].

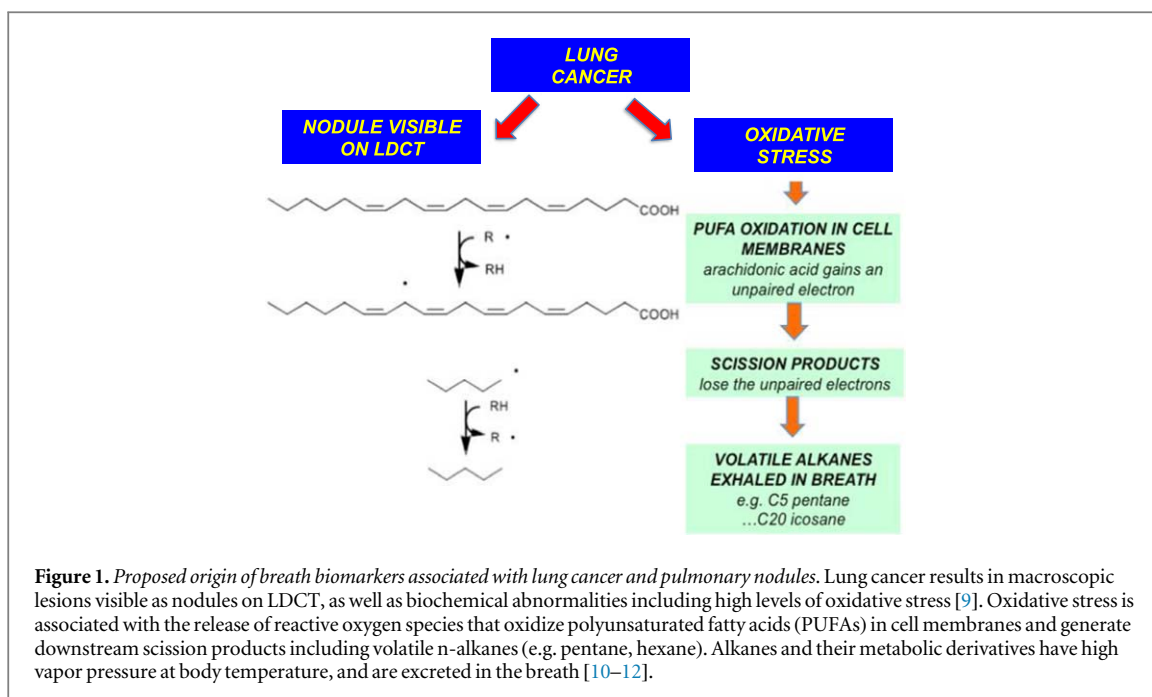


Figure 1. Proposed origin of breath biomarkers associated with lung cancer and pulmonary nodules. Lung cancer results in macroscopic lesions visible as nodules on LDCT, as well as biochemical abnormalities including high levels of oxidative stress [9]. Oxidative stress is associated with the release of reactive oxygen species that oxidize polyunsaturated fatty acids (PUFAs) in cell membranes and generate downstream scission products including volatile n-alkanes (e.g. pentane, hexane). Alkanes and their metabolic derivatives have high vapor pressure at body temperature, and are excreted in the breath [10–12].

This puzzling anomaly prompted us to test a more rigorous approach to biomarker identification and validation. The current approach to lung cancer detection employs primary screening with low-dose computerized tomography of chest (LDCT), followed by biopsy of lung nodules with a ‘suspicious’ appearance. The diagnosis is then confirmed by the microscopic appearance of lung cancer in the biopsy specimen. We hypothesized that a robust breath biomarker of lung cancer should therefore fulfill two independent criteria: it should be increased in patients who have suspicious lung nodules observed on LDCT, and also in patients who have biopsy-proven lung cancer (figure 1).

We report here a test of this hypothesis. We re-analyzed data from a recent study in order to determine if a breath biomarker could predict biopsy-proven lung cancer as well as lung nodules seen on low-dose computerized tomography of chest (LDCT). In order to maintain rigorous cross-validation, we employed data from two sequential clinical studies in order to identify the biomarker in the training set, and then test it in a blinded validation set.

Methods and materials

Human subjects

The clinical study has been reported [13]. We re-analyzed the breath VOC chromatograms performed in 462 subjects who had been screened with low-dose computerized tomography of chest (LDCT): 301 in the unblinded model-building phase (mean age = 61.8 yr, SD = 7.24), and 161 in the blinded model-testing phase (mean age = 62.0 yr, SD = 7.42). The blinded study was monitored with Good Clinical Practice.

Collection and analysis of VOCs in breath

The method has been described [5, 14]. Subjects wore a nose clip and respired normally through a disposable valved mouthpiece and bacterial filter into a breath collection apparatus for 2.0 min, and the VOCs in 1.0 l alveolar breath were captured on to a dual-bedded sorbent trap (Carbotrap C and Carbopack C, Supelco Inc, Bellefonte, PA). Duplicate breath VOC samples were collected from all subjects and stored in hermetically sealed containers at -15°C prior to replicate assays at two independent laboratories (Menssana Research, Inc and American Westech, Inc., Harrisburg, PA). Refrigerated samples are stable for at least 3 yr.

Samples were analyzed with automated thermal desorption gas chromatography mass spectrometry (ATD GC MS). A known quantity of bromofluorobenzene (BFB) internal standard was automatically loaded on to all samples in order to normalize the abundance of VOCs and to facilitate alignment of chromatograms. A computerized library (NIST Mass Spectral Library <http://nistmassspectralibrary.com>) was employed to assign a tentative chemical structure to each VOC based on the quality of fit with each VOC’s mass spectrum. This analytical method typically reveals approximately 200 different VOCs in a single sample of human breath.

Biomarker discovery and validation

Unblinded training set

GC MS chromatograms of breath VOCs were processed to generate a table of ion masses with their intensities and retention times normalized to BFB. Every mass ion was ranked as a candidate biomarker of a suspicious lung nodule observed on LDCT, and also of biopsy-proven lung cancer according to the area

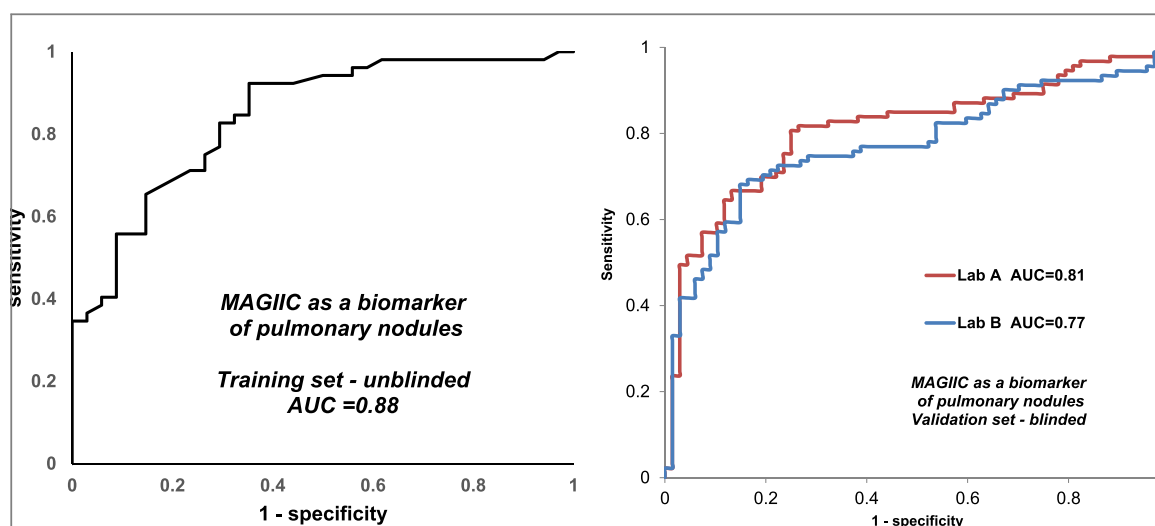


Figure 2. *MAGIIC as a biomarker of pulmonary nodules suspicious for lung cancer. Training set ROC curve (unblinded)—left panel.* The MAGIIC biomarker was discovered by statistical analysis of the unblinded dataset. This figure displays the receiver operating characteristic (ROC) curve of MAGIIC as a candidate biomarker of pulmonary nodules. The ‘gold standard’ of a pulmonary nodule was a radiologist’s report of a nodule observed on LDCT that was suspicious for lung cancer. The area under curve (AUC) of the ROC curve indicates 88% correlation accuracy. *Validation set ROC curve (blinded)—right panel.* Breath VOC samples were assayed at two independent laboratories that were blinded to the clinical outcomes, laboratory A (red, Messana Research) and laboratory B (blue, Westech). The MAGIIC biomarker predicted the presence of pulmonary nodules in LDCT with 81% and 80% accuracy at laboratory A and laboratory B respectively, as shown by the AUC of the ROC curves.

under curve (AUC) of its receiver operating characteristic (ROC) curve. We then employed multiple Monte Carlo simulations to select the mass ion biomarkers that identified pulmonary nodules and lung cancer with greater than random accuracy. The methods have been described [15, 16].

Blinded validation set

161 subjects were screened for lung cancer with LDCT, and also had their breath analyzed for VOCs (figure 3). Candidate biomarkers identified in the unblinded training set were cross-validated in breath VOC chromatograms of breath VOCs as predictors of biopsy-proven lung cancer and for suspicious pulmonary nodules respectively.

Results

Unblinded biomarker discovery phase

Monte Carlo analysis identified a breath mass ion biomarker that correlated with biopsy-proven lung cancer as well as with suspicious pulmonary nodules observed on LDCT. The biomarker was termed *Mass Abnormalities in Gaseous Ions with Imaging Correlates (MAGIIC)*. The ROC curve of MAGIIC demonstrated 80% accuracy as a biomarker of lung cancer (figure 4, left panel) and 88% accuracy as a biomarker of pulmonary nodules (figure 2, left panel).

Blinded biomarker testing phase

The ROC curves of MAGIIC demonstrated 84% accuracy as a biomarker of lung cancer, with sensitivity = 75.4% and specificity = 85.0% where the sum of sensitivity plus specificity was maximal (figure 4,

right panel). MAGIIC also predicted pulmonary nodules with 77% and 81% accuracy analyzed at two independent laboratories (figure 3, right panel). Table 1 displays the positive predictive values and the false-positive rates of MAGIIC and LDCT as predictors of lung cancer.

Chemical identity of MAGIIC (table 2)

The MAGIIC biomarker is a mass ion defined by two analytic parameters: its m/z value (mass to charge ratio) and its chromatographic retention time. The parent compound source of the biomarker is not known with certainty. The most likely parent compound VOCs were identified by the NIST library based on their mass spectra. Note that three compounds on the list (1,4-butanediol, 2-pentanamine-4-methyl, and 2-propanamine) are consistent with metabolites of butane, pentane, and propane respectively i.e. metabolites of the volatile alkane products of oxidative stress shown in figure 1.

Effect of tobacco smoking on MAGIIC

No significant correlation was found between the abundance of MAGIIC and pack-years of tobacco smoking in the set of all current and former smokers. Least squares linear correlation indicated wide random scatter ($r^2 = 0.01$).

Discussion

The main finding was that a single biomarker in breath accurately predicted two independent outcomes: lung cancer confirmed on a tissue biopsy, and suspicious pulmonary nodules observed on LDCT. MAGIIC

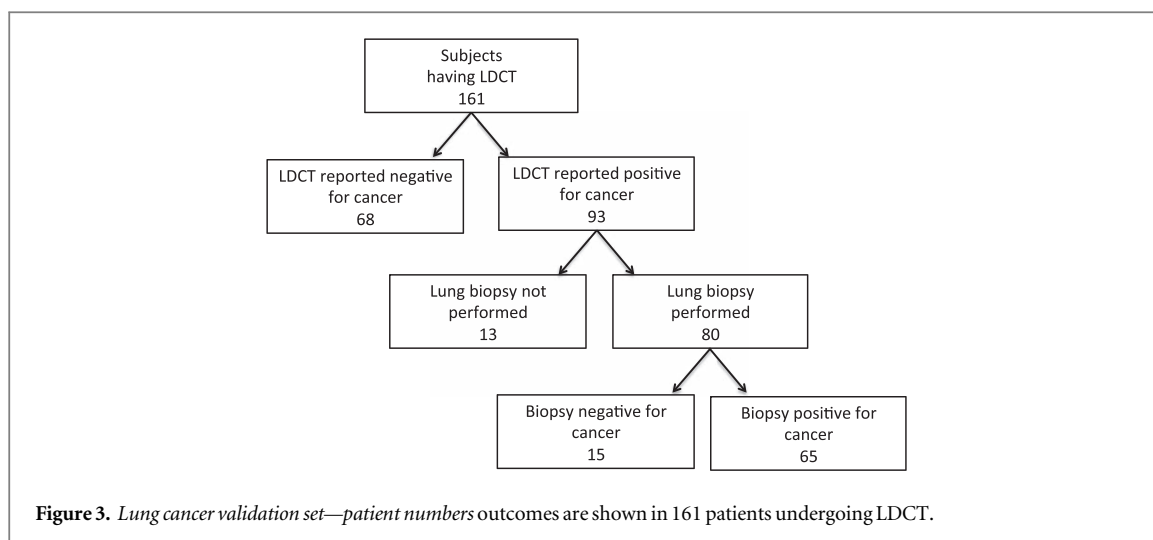


Table 1. Outcomes of MAGIIC and LDCT. Experimental outcomes are shown for LDCT and MAGIIC as predictors of biopsy-proven lung cancer in 161 screened subjects. TP = true positives, FP = false positives, TN = true negatives and FN = false negatives. The LDCT table employed values shown in figure 3, and MAGIIC employed sensitivity = 75.4% and specificity = 85.0 and % (figure 4, right panel).

		LDCT predicts cancer							
Cancer	On biopsy	positive	positive	negative	n/a	sensitivity %	n/a	PPV	FPR
			TP	FN					
		positive	65	FN				69.89	
		negative	FP	TN	68	specificity %	70.83	30.11	
		MAGIIC predicts cancer							
Cancer	On biopsy	positive	positive	negative	16	sensitivity %	75.38	PPV	FPR
			TP	FN					
		positive	49	FN				80.33	
		negative	FP	TN	68	specificity %	85.00	19.67	

Table 2. Identification of MAGIIC biomarker: The NIST library provided these candidate identifications of the chemical structure of MAGIIC based on the concordance of its mass spectrum with previously reported mass spectra of other compounds. These identifications should be regarded as tentative.

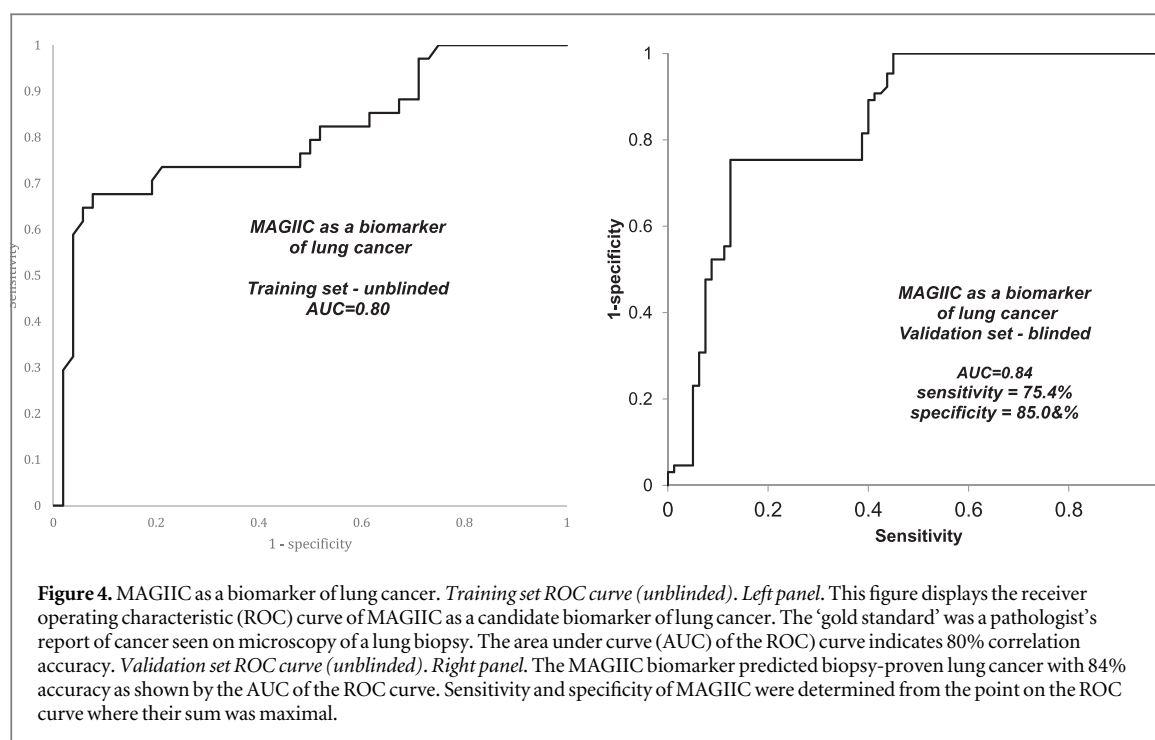
	CAS Registry Number
1,4-BUTANEDIOL	110-63-4
2-PENTANAMINE,4-METHYL-	108-09-8
2-PROPANAMINE	75-31-0
3-BUTENAMIDE	28446-58-4
4-PENTEN-2-OL	625-31-0
ACETAMIDE, 2-CYANOALANINE	n/a
N-METHYLGLYCINE	107-97-1
OCTODRINE	543-82-8

appeared to be a robust biomarker because it predicted two independent but related outcomes in a blinded study. The chemical structure of MAGIIC was not determined with certainty, but it was consistent with a volatile metabolite of alkane products of oxidative stress [10–12].

A MAGIIC breath test may have clinical applications as an ancillary to LDCT by providing information that could help guide physicians in reaching a decision to perform a lung biopsy. Small pulmonary nodules with diameters ≤ 10 mm are common LDCT

findings. Despite the traditional opinion that every pulmonary nodule should be considered malignant until proven otherwise, the majority of small lesions are benign, so that biopsy may not be necessary in all cases. CT-based methods have been developed to differentiate between benign and malignant lesions, including negative quantitative contrast-enhanced CT, and repeat nodule volume measurements at short-term follow-up CT examinations to detect growth suggestive of malignancy [17]. However, these additional imaging studies subject patients to potentially harmful radiation exposure as well as to increased costs. In clinical practice, the MAGIIC biomarker could potentially provide a useful tool for estimating the pre-test probability of the presence of lung cancer in a biopsy, analogous to the algorithms currently employed to predict the pre-test probability of an abnormal coronary angiogram [18, 19].

There is a clinical need to reduce the number of false-positive LDCT results. Annual LDCT is now widely employed for lung cancer screening, and Medicare and private insurers reimburse the test for asymptomatic smokers and ex-smokers aged 55–77 yr. However, the National Lung Screening Trial demonstrated that screening LDCT has potential harms as well as benefits [20–23]. 20.1% of all LDCTs yielded false-positive results, leading to over-investigation of



benign conditions. 5.5% suffered a major complication (e.g. pneumothorax) of an invasive procedure (e.g. bronchoscopy, mediastinoscopy).

In this study, we adopted a new strategy to minimize one of the central problems that has limited progress in breath research, a phenomenon termed 'voodoo correlations' i.e. biomarkers that are statistically significant but clinically meaningless [24]. Modern analytical tools such as GC MS regularly generate huge numbers of candidate biomarkers at comparatively low cost, but at the same time, the cost to recruit human subjects for clinical studies has increased dramatically. As a result, clinical studies may encounter increasingly large numbers of candidate biomarkers in comparatively small groups of human subjects. This increases the risk of over-fitting the data and generating 'voodoo correlations'.

In our previous report employing the same clinical dataset, we observed ~70 000 mass ions in chromatograms of breath. Multiple Monte Carlo simulations reduced this number to 544 mass ions that identified lung cancer with greater than random accuracy [13], and MAGIIC was observed as a member of this original set of candidate biomarkers of lung cancer. However, its accuracy (i.e. the AUC of its ROC curve) did not distinguish it from the other candidate biomarkers. The unique diagnostic value of MAGIIC in this dataset became apparent only when we tested a new hypothesis that a single biomarker should predict two conditions simultaneously i.e. pulmonary nodules as well as biopsy-proven lung cancer. This reduced the Universe of 70 000 candidate mass ions biomarkers to a small number in which MAGIIC delivered the best combination of sensitivity and specificity.

This approach could potentially be fruitful in future studies, by requiring a candidate biomarker to predict two related but independent conditions simultaneously e.g. a breath test for prostate cancer might evaluate biomarkers that simultaneously predict an abnormal MRI as well as the presence of cancer in a prostate biopsy.

We conclude that MAGIIC, a single volatile biomarker in the breath, accurately predicted lung cancer confirmed on a tissue biopsy, and suspicious pulmonary nodules observed on LDCT.

MAGIIC may have been a product of oxidative stress, and it could potentially be employed in clinical practice as ancillary to LDCT to predict the likelihood of a malignant nodule.

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