

Metabolic Signatures of Lung Cancer in Sputum and Exhaled Breath Condensate Detected by ¹H Magnetic Resonance Spectroscopy: A Feasibility Study

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ABSTRACT

OBJECTIVES: Lung cancer is one of the most lethal cancers. Currently, there are no biomarkers for early detection, monitoring treatment response, and detecting recurrent lung cancer. We undertook this study to determine if ¹H magnetic resonance spectroscopy (MRS) of sputum and exhaled breath condensate (EBC), as a noninvasive tool, can identify metabolic biomarkers of lung cancer.

MATERIALS AND METHODS: Sputum and EBC samples were collected from 20 patients, comprising patients with pathologically confirmed non-small cell lung cancer ($n = 10$) and patients with benign respiratory conditions ($n = 10$). Both sputum and EBC samples were collected from 18 patients; 2 patients provided EBC samples only. ¹H MR spectra were obtained on a Bruker Avance 400 MHz nuclear magnetic resonance (NMR) spectrometer. Sputum samples were further confirmed cytologically to distinguish between true sputum and saliva.

RESULTS: In the EBC samples, median concentrations of propionate, ethanol, acetate, and acetone were higher in lung cancer patients compared to the patients with benign conditions. Median concentration of methanol was lower in lung cancer patients (0.028 mM) than in patients with benign conditions (0.067 mM; $P = 0.028$). In the combined sputum and saliva and the cytologically confirmed sputum samples, median concentrations of *N*-acetyl sugars, glycoprotein, propionate, lysine, acetate, and formate were lower in the lung cancer patients than in patients with benign conditions. Glucose was found to be consistently absent in the combined sputum and saliva samples (88%) as well as in the cytologically confirmed sputum samples (86%) of lung cancer patients.

CONCLUSION: Absence of glucose in sputum and lower concentrations of methanol in EBC of lung cancer patients discerned by ¹H MRS may serve as metabolic biomarkers of lung cancer for early detection, monitoring treatment response, and detecting recurrence.

KEYWORDS: magnetic resonance spectroscopy, sputum, breath condensate, lung cancer

CITATION: Ahmed et al. Metabolic Signatures of Lung Cancer in Sputum and Exhaled Breath Condensate Detected by ¹H Magnetic Resonance Spectroscopy: A Feasibility Study. *Magnetic Resonance Insights* 2016;9 29–35 doi:10.4137/MRI.S40864.

TYPE: Original Research

RECEIVED: September 5, 2016. **RESUBMITTED:** October 19, 2016. **ACCEPTED FOR PUBLICATION:** October 25, 2016.

ACADEMIC EDITOR: Sendhil Velan, Editor in Chief

PEER REVIEW: Four peer reviewers contributed to the peer review report. Reviewers' reports totaled 1932 words, excluding any confidential comments to the academic editor.

FUNDING: We sincerely thank Department of Respiriology, Health Sciences Center, Winnipeg, for the funding support. The authors confirm that the funder had no influence over the study design, content of the article, or selection of this journal.

COMPETING INTERESTS: Authors disclose no potential conflicts of interest.

NOTE: This work was in part presented at the 24th Annual Meeting of the International Society of Magnetic Resonance in Medicine, Singapore (May 7–13, 2016).¹

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Introduction

Global lung cancer mortality is alarming. The primary reasons for the high mortality in lung cancer patients are as follows: lack of an established screening tool to detect early-stage lung cancer, advanced stage at presentation, and absence of biomarkers to monitor treatment response and to detect disease recurrence contributing to treatment failure and poor prognosis.^{2,3} Currently, implementing screening programs to detect early-stage lung cancer remains a challenge. In the National Lung Cancer Screening Trial (NLST), more than 50,000 participants underwent low-dose CT (LDCT) with 20% reduction in mortality with LDCT as compared to chest X-ray. However, LDCT triggered invasive diagnostic

procedures in 7% of the patients who did not have lung cancer. Considered as a low radiation dose procedure, the dose received by the participants who underwent LDCT was approximately 8 mSv per participant over three years. However, this translates into a risk of one death for screening every 2500 persons.⁴ Hence, there is an urgent need to explore noninvasive, pragmatic, and cost-effective tools to screen for early-stage lung cancer, which can complement and perhaps replace LDCT. It has been further suggested that analysis of biofluids (sputum, blood, and urine) might help identify subjects who should undergo LDCT.^{5–7}

Metabolomics research has opened new biological platforms to explore the disease processes, complementing

our existing knowledge of genomic, proteomic, and transcriptomic changes in a living cell.⁸ In the process of carcinogenesis, it has been postulated that cancer cells develop new biochemical adaptations with quantitative changes in endogenous metabolites.⁹ There has been a surge of investigators exploring magnetic resonance spectroscopy (MRS) and mass spectrometry (MS) techniques as noninvasive means to identify metabolomic signatures of cancer in body fluids and tissues.^{10–13} MRS can provide structural details of small organic molecules and can detect variations in the concentration of endogenous metabolites. It requires very little sample preparation and has several analytical advantages such as being nondestructive, quantitative, and reproducible.¹² It has been used to analyze body fluids in both malignant and benign conditions, including lung cancer, cystic fibrosis (CF), and chronic obstructive pulmonary disease (COPD).^{14–16}

We undertook the current pilot study to determine whether ¹H MRS can identify lung cancer-specific metabolic biomarkers in the sputum and exhaled breath of patients with known lung cancer. The hypothesis in question, in this study, is based on our previous work where we demonstrated that there was a relative “absence of glucose” in the sputum of lung cancer patients compared to those with benign conditions.¹⁷

Materials and Methods

Patients. Informed consent was obtained from each subject, and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki as reflected in a priori approval by the Institution’s Human Research Ethics Board. Sputum and exhaled breath condensate (EBC) samples were prospectively collected according to a preapproved protocol from 22 patients. Characteristics of the patients are summarized in Table 1. Patients with pathologically confirmed non-small cell lung cancer (NSCLC, $n = 12$), stage III–IV, before any oncologic treatment, and patients with respiratory conditions other than lung cancer (controls, $n = 10$) were enrolled at the respiratory clinic, University of Manitoba. The patients in the control group were confirmed not to have any clinical, radiological, and/or pathological evidence of lung cancer or any other malignant conditions. One patient with undetermined pathology and one with B-cell lymphoma were excluded from the analysis. Both sputum and EBC samples were collected from 18 patients; two patients provided EBC samples only. All specimens were collected by a dedicated respiratory research nurse and a respiratory technician in the institution’s pulmonary function laboratory. Specimens were stored at -80°C immediately after collection and later transported (on dry ice) to the University of Winnipeg for MRS analysis.

Specimen collection. Induced sputum samples (2–5 mL) were obtained from patients by inhalation of hypertonic saline solution. EBC was collected noninvasively by having the patient exhale into a cooled collecting container and breathing comfortably through a mouthpiece with a nose clip in place (to prevent loss of exhaled volume through the nose). Approximately

Table 1. Clinical characteristics of the patients enrolled in the study.

CHARACTERISTICS	PATIENTS WITH NSCLC % (n)	PATIENTS WITH BENIGN CONDITIONS % (n)
Gender (male:female)	40:60	80:20
Age mean (STD)	68.3 (10.0)	63.0 (13.3)
COPD	40 (4)	70 (7)
Associated respiratory infection	30 (3)	29 (2)
Diabetes	0	10 (1)
Previous cancer	40 (4)	22 (2)
Steroid user	10 (1)	56 (5)
Current smoker	0	22 (2)
Asbestosis	0	11 (1)
Histopathology	Adenocarcinoma: 50 (5)	
	Squamous cell carcinoma: 50 (5)	
Stage	III: 40 (4), IV: 50 (5), unknown: 10 (1)	
Tumor location	Central: 60 (6); peripheral: 40 (4)	
Other conditions	0	
Churg strauss vasculitis	0	10 (1)
Granulomatous inflammation	0	10 (1)
Sarcoidosis	0	10 (1)
Neurofibromatosis	0	10 (1)
Exudative pleural effusion	0	10 (1)

2–3 mL of EBC was collected from each patient over a period of 10–20 minutes. A custom-built condenser with a metal element surrounding a tightly fit inner collection tube was used for this purpose (Fig. 1). Both our collecting device and the technique followed the methodological recommendations set by the American Thoracic Society and European Respiratory Society Task Force published in the *European Respiratory Journal*.¹⁸

Chemicals. Deuterium oxide (D_2O), sodium chloride (NaCl), PBS buffer tablets, and 3-(trimethylsilyl) propionic-2,2,3,3- d_4 acid sodium salt (TSP) were purchased from Sigma-Aldrich.



Figure 1. Exhaled breath condensate (EBC) collection device with a nasal clip.



Table 2. Metabolites identified in EBC samples.

METABOLITE	LUNG CANCER	CONTROL GROUP (BENIGN CONDITIONS)	P
Propionate	0.022 (0–0.060)	0.012 (0–0.077)	0.94
Ethanol	0.24 (0.14–0.43)	0.19 (0.14–0.36)	0.53
Acetate	0.16 (0.09–0.22)	0.11 (0.08–0.20)	0.53
Acetone	0.026 (0.013–0.039)	0.023 (0–0.049)	0.79
Methanol	0.028 (0.023–0.057)	0.067 (0.043–0.09)	0.028

Notes: Values are presented as median (interquartile) concentrations (mM). Samples (continuous data) are compared using the Wilcoxon two-sample test.

MRS data collection and analysis. MRS staff were blinded to the clinical characteristics and the pathological diagnoses of the patients.

For sputum analysis, samples were thawed for 10–15 minutes, and ~300 µL 2M NaCl solution (buffered with PBS/D₂O, pH = 7.4) was added to each sample in 1:1 ratio (v/v), mixed by vortex to get homogenous clear suspensions. Frozen EBC samples were thawed, and neat samples were used for analysis. All MRS experiments were performed on a Bruker Avance 400 MHz NMR spectrometer at the University of Winnipeg. A volume of 500 µL of the processed sputum or EBC sample was transferred into a 5-mm NMR tube along with separate reusable coaxial capillary tubes containing a standard solution of TSP prepared in D₂O (TSP concentration = 1.538 and 1.763 mM for sputum and EBC samples, respectively). TSP served as both chemical shift reference for aligning the spectra (0.0 ppm) and external concentration reference, whereas D₂O was used as a “deuterium lock”. One-dimensional (1D) ¹H MRS experiments with single pulse or excitation sculpting (ES) pulse sequence were performed on all samples with no spinning at 25°C. The following acquisition

parameters were employed in all 1D experiments: number of scans = 32, a 45° pulse (single pulse: zgpr) and a 90° pulse (for ES: zgesgp), number of points in time domain = 32 k, interpulse delay = 5 seconds, spectral width = 5556 Hz, acquisition time = 2.95 seconds, and line broadening for exponential window function = 0.3 Hz. When using excitation sculpting sequence, the power of the shaped pulse (sp1) was optimized to get a better suppression of water signal. For more details on the acquisition parameters, see the Appendices 1 (zgpr) and 2 (zgesgp).

Various metabolite peaks were confirmed by performing ¹H-¹H COSY or TOCSY experiments on representative samples. Moreover, we have also compared ¹H chemical shifts of metabolites with those from NMR databases such as the human metabolome database (HMDB).¹⁹ The peak areas of the signals from metabolites of interest (Tables 2 and 3) were measured by manual integration using TSP as an external reference. Concentrations of various metabolites were calculated as previously shown by Ijare et al.²⁰

Cytological confirmation of the sputum samples. An independent dedicated lung pathologist analyzed sputum samples for cytological confirmation of sputum. Presence or absence of alveolar macrophages differentiated between sputum and saliva. The pathologist was blinded to the clinical characteristics and the observed metabolic profiles of the collected specimens.

Statistical analysis. Dichotomous outcomes were compared using Fisher’s exact test. Continuous data were analyzed with the Wilcoxon two-sample test. SAS9.4© software was used for the statistical analyses.

Results

¹H MRS of EBC samples (Fig. 2) showed the presence of metabolites such as propionate, ethanol, acetate, acetone, and methanol. Propionate, ethanol, acetate, and acetone were

Table 3. Metabolites in sputum samples without cytological confirmation (sputum and saliva) and with cytological confirmation (true sputum).

METABOLITES IN SPUTUM	WITHOUT CYTOLOGICAL CONFIRMATION		P	WITH CYTOLOGICAL CONFIRMATION	
	LUNG CANCER	CONTROL GROUP (BENIGN CONDITIONS)		LUNG CANCER	CONTROL GROUP (BENIGN CONDITIONS)
Glycoprotein	1.58 (1.17–2.62)	2.28 (1.74–3.43)	0.14	1.76	3.43
Propionate	0.46 (0.42–0.92)	0.59 (0.29–0.88)	0.90	0.46	0.58
Lactate	0.52 (0.38–0.83)	0.50 (0–0.75)	0.69	0.51	0.64
Acetate	2.40 (1.24–5.40)	2.69 (1.71–4.17)	0.9	2.21	2.53
N-acetyl sugars	3.46 (3.08–4.74)	5.09 (4.35–8.39)	0.1	3.58	8.39
Lysine	1.23 (0.48–1.48)	1.46 (0.69–1.89)	0.19	1.35	1.89
Choline	0 (0–0.10)	0.03 (0–0.16)	0.77	0	0.16
Formate	0.20 (0.04–0.33)	0.22 (0.12–0.28)	0.63	0.13	0.19
% of subjects with absent glucose	88%	30%	0.59	86%	40%

Notes: Values are presented as median (interquartile) concentrations (mM). Samples (continuous data) are compared using the Wilcoxon two-sample test except for glucose where data were compared with Fisher’s exact test since the data were dichotomous.

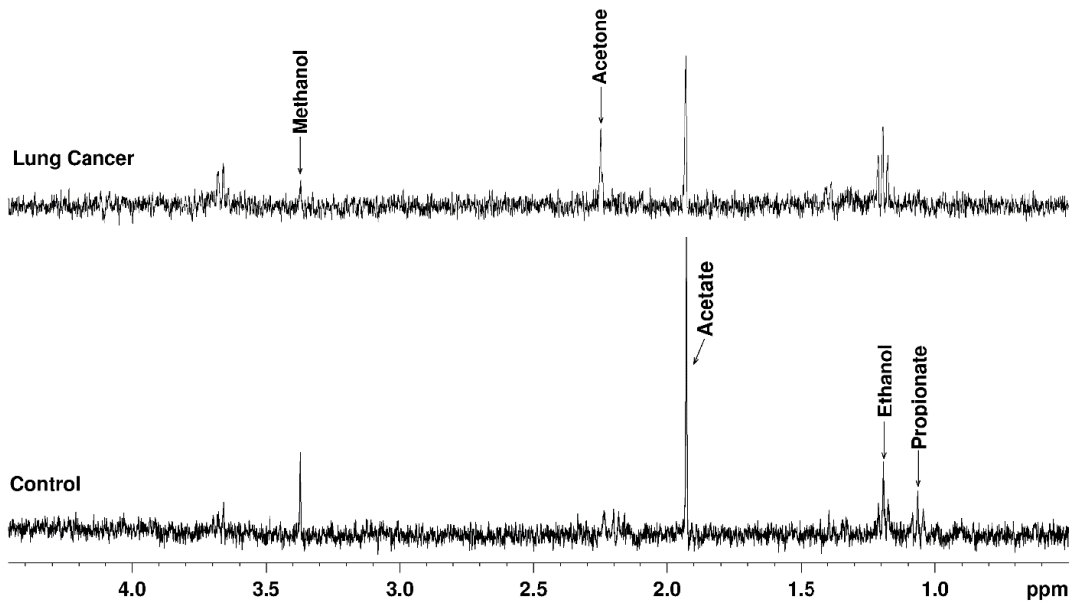


Figure 2. ¹H MR spectra (ES sequence) of exhaled breath condensate (EBC) samples from control and lung cancer patients (adenocarcinoma) showing relative levels of metabolites.

elevated in lung cancer patients compared to the patients with benign conditions. The median concentration of methanol was found to be lower in lung cancer patients (0.028 mM) than in patients with benign conditions (0.067 mM; $P = 0.028$). However, the difference in the median concentrations of other metabolites between the two groups was not statistically significant (Table 2). Furthermore, subset analysis based on the tumor location in cancer group revealed that the median

concentration of methanol from tumors in the central location (6/10) was lower compared to the peripheral location (4/10), but the difference was not statistically significant (0.028 vs. 0.032 mM; $P = 0.76$).

Cytology of the sputum samples revealed that 7 of 8 lung cancer patients and 5 of 10 patients with benign diseases had cytologically confirmed sputum. ¹H MRS of sputum samples (Fig. 3) showed the presence of *N*-acetyl

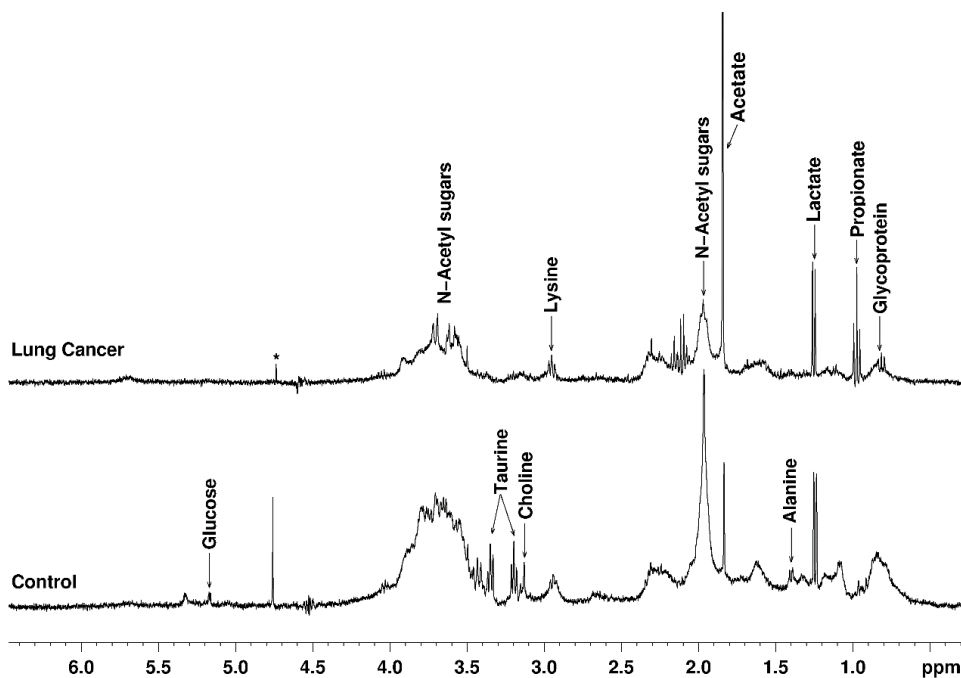


Figure 3. ¹H MR spectra (ES sequence) of sputum samples from a control subject and a lung cancer patient (adenocarcinoma) showing relative levels of metabolites including the absence of glucose in lung cancer patient (*residual water signal).



sugars, glycoprotein, propionate, lysine, acetate, and lactate. Median concentration of these metabolites was lower in the lung cancer patients compared to patients with benign conditions. Glucose was absent in six of seven (86%) lung cancer patients and two of five (40%) patients with benign diseases (Table 3). Median concentration of metabolites in samples without cytological confirmation of sputum (sputum plus saliva) was also obtained from both groups of patients (Table 3).

Discussion

The process of carcinogenesis alters the metabolism of the cells due to genetic aberrations in their normal signaling pathways.²¹ Newly developed malignant cells undergo a metabolic transformation by generating ATP, macromolecules, and altering the impact of reactive oxygen species to support their proliferation. As a consequence, tumor microenvironment manifests hypoxia, alteration in its pH, and nutritional status.^{21,22} MRS or MS has been used to identify altered metabolic environment of cancer cells in different body fluids.¹² In this study, using a home-built EBC-collecting device, it was feasible and convenient to obtain EBC samples from all the patients. ¹H MRS identified specific compounds (propionate, ethanol, acetone, and acetate) with higher concentration in cancer patients; however, the median concentration of methanol was 41% lower in EBC samples of cancer patients compared to the control group ($P = 0.028$). In a recent review, based on the studies carried out during 1985–2015, the most frequent volatile organic compounds (VOCs) identified as biomarkers in the breath of lung cancer patients were 2-butanone, 1-propanol, isoprene, ethylbenzene, styrene, and hexanal. These biomarkers were identified mostly by using gas chromatography in combination with mass spectrometry (GC-MS).²³ However, in this review, the largest series included 220 lung cancer patients and 441 healthy volunteers using proton transfer reaction mass spectrometry (PTR-MS). This study reported lower median concentration of methanol in the breath samples of lung cancer patients than healthy controls; 118.5 vs. 142.0 ppb; ($P = 0.011$).²⁴ Interestingly, methanol is present in very small amounts in the breath of healthy adults with a concentration in the range of 32–2319 parts-per-billion by volume. It is primarily produced in the gut by the interaction of bacteria with the unabsorbed carbohydrates and can be detected in body fluids including blood, urine, and breath condensate. Ingested fruits, vegetables, and aspartame can effect the endogenous production of methanol and hence its level of concentration in body fluids.^{25–28} So far, there has not been any previous report explaining the altered metabolism of methanol in malignant cells. Significantly reduced levels of methanol in the breath of lung cancer patients observed in this study and in the study by Bajtarevic et al²⁴ indicated a possible alteration in its metabolic pathway triggered by the malignant transformation of the normal

cells and warrants further investigation. Moreover, it is also worth noting in our study that patients with central lesions either in close proximity or involving the tracheobronchial tree with or without a lymph node mass had lower methanol concentration as compared to peripheral lesions. The difference, however, is not statistically significant possibly due to the small sample size. Future studies with larger sample size may be able to differentiate the metabolic profile of tumors based on their location and tumor burden.

Sputum analysis with and without cytological confirmation has consistently demonstrated absence of glucose in most of the pathologically confirmed lung cancer patients enrolled in our study. This is a novel finding only reported by our group previously.¹⁷ Interestingly, some of the other metabolites (specifically glycoproteins and *N*-acetyl sugars) in both sets of samples—combined sputum and saliva and cytologically confirmed true sputum samples—also consistently revealed lower median concentrations in the lung cancer group (Table 3). However, since only 50% of the samples from the benign diseases group had cytologically confirmed sputum, we remain uncertain about the observed metabolic profile in this group. It is also worthwhile to note that there were two non-cancer patients who had cytologically confirmed sputum samples but whose spectra did not show the presence of glucose signal. One of them was a diabetic patient and currently on metformin. The other patient had increased glucose level in the blood around the period of sample collection.

The authors acknowledge that sputum could not be obtained from all the patients. However in spite of the difficulties in obtaining induced sputum samples, sputum, as a surrogate of altered metabolism of the malignant process, is emerging as an important noninvasive tool to diagnose benign diseases and lung cancer. Guzmán et al have demonstrated methylation of tumor suppressor genes in sputum to diagnose lung cancer and COPD.²⁹ Another group recently has discovered a set of metabolites in the sputum of lung cancer patients with flow infusion electrospray ion MS.³⁰ Such metabolic phenotype may be an outcome of proliferating cancer cells that can reprogram their own metabolic activity or enzymatic metabolic pathways through activated oncogenes and inactivated tumor suppressor genes at mitochondrial level.^{31–33} Cancer cells initiate anaerobic pathway for their survival even in the presence of oxygen and generate energy through glycolysis, previously described as Warburg effect.^{11,34} With metabolic transformation of the cells, mitochondrial oxidative phosphorylation is suppressed and replaced by glycolysis and accumulation of lactate, providing a more efficient form of ATP production for the rapidly proliferating tumor cells.³⁵ However, compromised mitochondrial activity and enhanced glycolysis in proliferating cancer cells may not be the entire story. The two sources of energy; oxidative phosphorylation in mitochondria and glycolysis may operate preferentially and



under different conditions of the tumor microenvironment including hypoxia and rate of tumor growth. Oncogenes and associated proteins such as HIF-1 α , RAS, C-MYC, SRC, and p53 can also influence the glycolytic pathway and the process of oxidative phosphorylation.³⁶ Gao et al³⁷ have demonstrated the role of C-MYC regulating the glutamine metabolism at mitochondrial level providing ATP for the proliferating lymphoma and prostate cancer cells. Reduced availability of glucose in the sputum of lung cancer patients as demonstrated in our study is intriguing and novel. It may provide a new dimension of the altered metabolism specifically in the context of lung cancer.

Limitations of Our Study

This was a pilot study with a small sample size. It was not possible to correlate several clinical variables including smoking, COPD, diabetes, age of the patient, stage of the cancer, drug profile location of the disease, and molecular markers (EGFR, ALK) with the identified metabolites. We also acknowledge that there were fewer samples of cytologically confirmed sputum in the group with benign diseases that makes statistical evaluation difficult. We need to find novel techniques to improve our ability to obtain induced sputum more reliably.

Conclusion

Lower levels of methanol in EBC samples and the absence of glucose in sputum of lung cancer patients detected by MRS is novel and significant, warranting further investigation in a larger clinical study.

Acknowledgment

We also thank Ann Szabo and Garth Rodgers for their technical assistance.

Author Contributions

Conceived and designed the experiments: NA, TB, OI, RM, SB, and ZB. Analyzed the data: ZN, NA, OI, and RA. Wrote the first draft of the manuscript: NA, OI, and TB. Contributed to the writing of the manuscript: JK and GQ. Agree with manuscript results and conclusions: NA, TB, OI, RM, RA, MA, ZB, SB, ZN, JK, and GQ. Jointly developed the structure and arguments for the paper: NA and OI. Made critical revisions and approved final version: NA, OI, TB, and MA. All authors reviewed and approved of the final manuscript.

Supplementary Materials

Appendix 1. “ased” output of pulse sequence “zgpr” used for collecting ¹H NMR data on sputum and EBC samples.

Appendix 2. “ased” output of pulse sequence “zgesgp” used for collecting ¹H NMR data on sputum and EBC samples.

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