

# Proton Magnetic Resonance Spectroscopy of Sputum for the Non-Invasive Diagnosis of Lung Cancer: Preliminary Findings

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**Abstract:** *Aims and Background:* Sputum has been examined for the identification of potential biomarkers for the non-invasive diagnosis of lung cancer. However, no definitive biomarkers with reliable accuracy have been identified yet. The main objective of this work was to evaluate the utility of magnetic resonance spectroscopy (MRS) in the analysis of sputum for the non-invasive diagnosis of lung cancer.

*Methods:* Induced sputum samples from lung cancer patients (n = 9) and control subjects (n = 6) were collected for proton (<sup>1</sup>H) MRS analysis. Samples from two cancer patients and one control subject were discarded as these samples were confirmed to contain only saliva by cytologic examination. Only the true sputum specimens containing alveolar macrophages were analyzed by <sup>1</sup>H MRS. To facilitate MRS analysis, sputum samples were dispersed in 2M sodium chloride solution buffered with phosphate-buffered-saline (PBS). MR spectra were obtained using a one-pulse sequence with presaturation of the water resonance.

*Results:* Glucose was found to be absent in sputum samples obtained from lung cancer patients. Spectra of sputum samples collected from control subjects showed presence of glucose signal except for one whose sputum cytology indicated the presence of atypia. The absence of glucose in sputum from cancer patients could be attributed to an increased rate of glycolysis in the lung cancer cells. The present observation, albeit on a small sample size, showed a better sensitivity (100%) and overall accuracy (92%) compared to sputum cytology (sensitivity = 50%; overall accuracy = 70%).

*Conclusions:* Absence of glucose in sputum could be an indicator of lung cancer and the present methodology can be a valuable addition to the non-invasive diagnostics of lung cancer.

**Keywords:** Cytology, glucose, lung cancer, magnetic resonance spectroscopy, sputum analysis.

## INTRODUCTION

Lung cancer is the leading cause of cancer-related death, among both men and women [1,2]. In 2012, the National Cancer Institute estimates 226, 160 new cases with an estimated deaths of about 160, 340 in the USA [2]. Despite advances in the treatment, the average 5-year survival rate for lung cancer is only 15%. One of the reasons for the low survival rate is the asymptomatic nature of the disease in the early stage. Recently, the National Lung Screening Trial (NLST) research team revealed that screening persons at high risk for lung cancer with low-dose computed tomography (LDCT) has reduced lung cancer mortality by 20% [1]. Further, they suggested the analysis of biospecimens (blood, sputum and urine) may help identify persons who should undergo LDCT and also select those subjects with positive LDCT results who will need further diagnostic evaluation [1]. Hence, there is a strong rationale for developing screening methods using site-specific biospecimens such as sputum.

Currently available diagnostic techniques for the detection of lung cancer include imaging methods

(chest X-ray, CT, PET, MRI), bronchoscopy, and sputum cytology. Molecular screening methods such as analysis of blood, sputum, bronchoalveolar lavage and exhaled breath are also being explored [3]. Sputum cytology is currently the only non-invasive method that can detect pre-malignant lesions or carcinoma *in situ* in the tracheobronchial tree. However, it has an average sensitivity of only 61% [4]. Sputum has been considered as a potential source of biomarkers for the detection of lung cancer. Recently, there has been some work on identifying genetic markers (*ENO1*, *FHIT*, *HYAL2*, *SKP2*, *p16*, and *14-3-3zeta*) in sputum samples for the early diagnosis of lung cancer [5]. However, no definitive biomarkers have been identified to date [6]. Although MRS has been extensively used in metabolomics-based studies in the analysis of biofluids/cell homogenates/tissue extracts, there are limited reports on the use of MRS in the analysis of sputum and saliva for diagnostic applications [7-9]. In this study, we have tested the feasibility of MRS-based sputum analysis for the non-invasive diagnosis of lung cancer.

## MATERIALS AND METHODS

### Patients

Induced sputum samples were collected (by inhalation of hypertonic saline solution) from 15

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subjects (non-small cell lung cancer, NSCLC, n=5; small cell lung cancer, SCLC, n=3; adenocarcinoma, n=1; normal controls, n=6) at the Ottawa Hospital. Samples were frozen at  $-70^{\circ}\text{C}$  and shipped on dry ice to NRC Institute for Biodiagnostics, Winnipeg for MRS analysis. Samples from two cancer patients and one control subject were discarded as these samples were confirmed to be only saliva on cytologic examination. Only the true sputum specimens (cancer, n = 7; control, n = 5) were included in this study. Split aliquot portions of the samples were used for cytologic examination. Saliva samples obtained from 2 volunteers were used to differentiate the  $^1\text{H}$  MRS spectral patterns of sputum from saliva. Medical diagnoses of the cancer patients were based on the detailed histology of the lung tissue specimens obtained during surgery. Informed consent was obtained from each subject and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in *a priori* approval by the Institutions' human research ethics boards.

### Chemicals

Phosphate buffered saline (PBS), glucose, 3-(trimethylsilyl)propionic-2,2,3,3- $\text{d}_4$  acid sodium salt (TSP), and deuterium oxide ( $\text{D}_2\text{O}$ ) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

### Preparation of Sputum Suspensions

The sputum samples were thawed in a biological safety cabinet for 10-15 min, and  $\sim 300\ \mu\text{L}$  2M NaCl solution (buffered with PBS/ $\text{D}_2\text{O}$ , pH= 7.4) was added to each sample in 1:1 ratio (v/v), mixed by vortex to get homogenous clear suspensions.

### Cytology Preparation

Each sample of sputum was prepared using the "pick and smear" technique, where fresh sputum samples were examined for tissue fragments and blood. Two smears were prepared for each case from areas that contained these elements and immediately fixed in 95% ethanol. The smears were then stained with Papanicolau stains and observed under low-power microscope to detect the presence/absence of pulmonary macrophages and squamous epithelial cells (SECs).

### MRS Experiments

All MRS experiments were performed on a Bruker Avance 360 MHz NMR spectrometer. A volume of

$500\ \mu\text{L}$  of sputum suspension was transferred to a 5-mm NMR tube along with a reusable co-axial capillary tube containing TSP in  $\text{D}_2\text{O}$  (0.0965 mg TSP/ $150\ \mu\text{L}$   $\text{D}_2\text{O}$ ). TSP served as a chemical shift reference (0.0 ppm) whereas  $\text{D}_2\text{O}$  was used as a 'deuterium lock'.  $^1\text{H}$  MRS spectra of all samples were recorded with no spinning at  $25^{\circ}\text{C}$ . One dimensional (1D)  $^1\text{H}$  MRS experiments using one-pulse sequence were performed with presaturation of water resonance. The following acquisition parameters were employed in all 1D experiments: NS (number of scans) = 128, P1 ( $90^{\circ}$  pulse) =  $5.93\ \mu\text{s}$ , PL9 (water presaturation power) = 60 dB, TD (number of points in time domain) = 32k, D1 (interpulse delay) = 5 s, SW (spectral width) = 5800 Hz, AQ (acquisition time) = 2.85 s and LB (line broadening for exponential window function) = 0.3 Hz.

### Statistical Analysis

Sensitivity, specificity and overall accuracy were calculated for both MRS-based diagnosis and sputum cytology, and both results were compared.

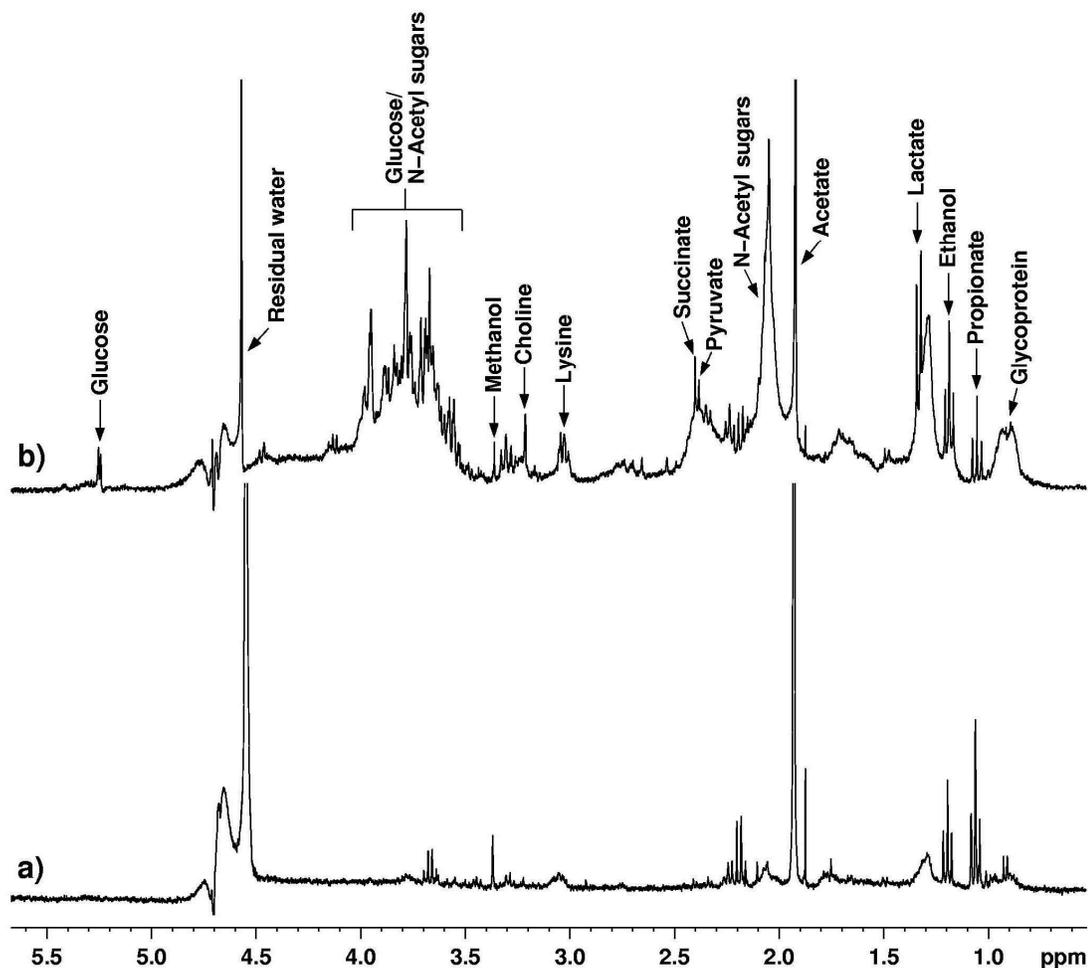
## RESULTS

### Cytology

Sputum consists of a mixture of cellular and non-cellular elements that are cleared by the mucocilliary apparatus. Samples were considered to be true sputum if they had  $>25$  pulmonary macrophages. Specimens consisting of only SECs are considered unsatisfactory because they represent only saliva, and are excluded from the study. Of the 15 samples collected, three samples were unsatisfactory. As per cytology, the diagnoses of the remaining 12 samples were: small cell carcinoma (2 cases), cytologic atypia (2 cases) and negative for malignancy (8 cases).

### $^1\text{H}$ MRS

In order to reduce its viscosity and facilitate the MRS analysis, we dispersed the sputum in 2M NaCl solution. Figure 1 shows typical 1D  $^1\text{H}$  MR spectra of (a) saliva (from a healthy volunteer) and (b) sputum (from a control subject). Some common biochemicals detected in both samples have been marked in Figure 1. The assignments in the saliva samples were consistent with those reported in the literature [7,9]. Both saliva and sputum samples were characterized by the presence of propionate, ethanol, lactate, acetate, N-acetyl sugars, pyruvate, succinate, lysine, choline, methanol and formate. It is interesting to note that glucose was detected only in sputum samples but not



**Figure 1:**  $^1\text{H}$  MR spectra (360 MHz) of (a) saliva (from a healthy volunteer) and (b) sputum (from a control subject), both dispersed in 2M NaCl solution (buffered with PBS/D<sub>2</sub>O, pH = 7.4), showing spectral similarities/differences.

in saliva. N-acetyl sugars and a few unidentified broad signals (probably arising from glycoproteins such as mucin) in the region 0.8 – 1.0 ppm and 1.2 – 1.4 ppm were observed in elevated levels in sputum samples. Sputum is secreted by the lower respiratory tract (LRT)/tracheobronchial tree under inflammatory conditions and is characterized by the presence of polymers such as DNA, proteoglycans, and biofilms along with inflammatory cells such as alveolar macrophages [10]. Contamination of sputum with URT secretions is a major problem in the sample collection. However, such contaminations could be detected by observing the presence of SECs in sputum samples which are predominantly observed in URT/saliva.

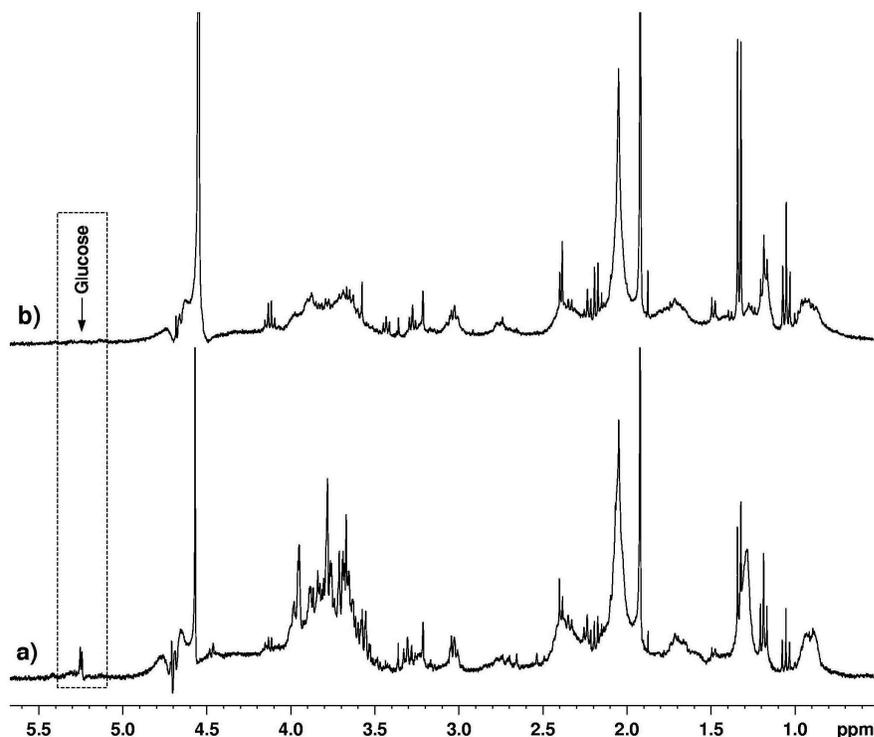
Figure 2 shows typical 1D  $^1\text{H}$  MR spectra of sputum samples from (a) control and (b) lung cancer subjects. When comparing the spectral patterns of the sputum samples from both groups, we can see that the control subject shows the presence of glucose, whereas glucose was absent in the lung cancer patient.

Interestingly, glucose was absent in all of the seven patients with lung cancer. Glucose was also absent in one of the control subjects (see Table 1 for details). However, the sputum cytology report for this subject was found to be cytologic atypia which can be considered to be a high risk for the development of lung cancer [11].

We have compared the results of the present MRS study with sputum cytology analysis. The present method showed a sensitivity of 100% and specificity of 80% with an overall accuracy of 92% whereas sputum cytology showed a sensitivity of 50%, specificity of 100% with an overall accuracy of 70%.

## DISCUSSION

The reason for the absence of glucose in sputum samples obtained from the lung cancer patients could be due to an increased rate of glycolysis in the cancer cells [12]. Cancer cells adapt to glycolytic pathway for



**Figure 2:**  $^1\text{H}$  MR spectra (360 MHz) of sputum dispersed in 2M NaCl solution buffered with PBS/D<sub>2</sub>O, pH = 7.4 from (a) a control subject and (b) a lung cancer patient. Note that glucose is absent in the sputum of lung cancer patient. Please refer to Figure 1 for peak assignments.

**Table 1: Clinical Details of Subjects with/without Lung Cancer Along with Sputum Cytology and  $^1\text{H}$  MRS Results**

Patient #	Age/ Gender	Smoking history	Medical Diagnosis (Histology)	Stage	Sputum cytology	$^1\text{H}$ MRS pattern	Sputum- glucose by $^1\text{H}$ MRS
1	78/M	NA	Non-cancer	-	Negative	Sputum	Present
2	32/M	NA	Non-cancer	-	Negative (mostly saliva)	Sputum mixed with saliva	Present
3	30/F	Smoker	Non-cancer	-	Negative	Sputum	Present
4	48/M	Smoker	Non-cancer	-	Negative	Sputum	Present
5	31/F	N-smoker	Non-cancer	-	Atypia	Sputum	Absent
6	78/M	F-smoker	SCLC	IV	SCLC	Sputum	Absent
7	62/F	F-smoker	SCLC	IIIB	Negative (mostly saliva)	Sputum mixed with saliva	Absent
8	68/M	Smoker	SCLC	IIIB	SCLC	Sputum	Absent
9	85/M	F-smoker	NSCLC	IB	Negative	Sputum	Absent
10	79/F	Smoker	NSCLC	IIIA	Negative	Sputum	Absent
11	53/F	Smoker	NSCLC	IV	Negative	Sputum	Absent
12	66/M	F-smoker	ADC	IV	Suspected malignancy	Sputum	Absent

Abbreviations: NA, not available; N-smoker, Non-smoker; F-smoker, Former smoker; SCLC, small cell lung cancer; NSCLC, non small cell lung cancer; ADC, adenocarcinoma.

the generation of ATP to meet their energy needs, and as a result, they utilize glucose at higher rates than the normal cells (the Warburg effect) [13,14]. In normal cells, generation of ATP through oxidative phosphorylation in the mitochondria is a preferred

metabolic pathway (Krebs cycle). Since cancer cells have defective Krebs cycle, they adapt alternative metabolic pathways and rely entirely on glycolysis to maintain their energy needs [14]. Moreover, the glycolytic ATP production requires availability of  $\text{NAD}^+$

which is supplied in cancer cells by the oxidation of NADH during the reversible transformation of pyruvate to lactate [15]. This transformation is catalyzed by a key enzyme, lactate dehydrogenase-5 (LDH-5), which is generally overexpressed in lung cancer patients [15]. In the present study, we have observed the presence of lactate in both controls and cancer patients, and could not see a correlation between the absence of glucose and the levels of lactate. Similarly, no other metabolites showed a positive correlation in differentiating cancer samples from the controls. This may be due to the limited size of the patient cohort. A multivariate analysis on MRS data obtained from a larger sample size would be able to identify additional biomarkers in addition to glucose.

It can be inferred from the present study that  $^1\text{H}$  MRS will have a potential in the non-invasive diagnosis of lung cancer. However, care should be exercised when collecting sputum samples. As saliva does not show the presence of glucose, minor sampling errors may lead to misdiagnosis. As shown in this study, the  $^1\text{H}$  MRS spectral patterns of saliva and sputum can be differentiated. Moreover, sputum cytology can be augmented with the present methodology for identifying contamination of sputum samples with saliva/URT secretions.

## CONCLUSION

The absence of glucose in the sputum samples from lung cancer patients may have a diagnostic potential. Although this observation is very promising, it needs to be validated on a large patient-cohort. Furthermore, multivariate analysis on MRS data obtained from sputum samples may be able to identify additional biomarkers in addition to glucose. Upon further validation, MRS of sputum may augment present screening/diagnostic modalities in the early and non-invasive diagnosis of lung cancer.

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