Lung Cancer 94 (2016) 88–95

Contents lists available at ScienceDirect

Lung Cancer

journal homepage: www.elsevier.com/locate/lungcan

The metabolomic detection of lung cancer biomarkers in sputum

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ARTICLE INFO

Article history:
Received 16 September 2015
Received in revised form 4 February 2016
Accepted 6 February 2016

Keywords:
Lung cancer
Metabolomics
Biomarkers
Sputum
Polyamines
Gangliosides

ABSTRACT

Objectives: Developing screening and diagnosis methodologies based on novel biomarkers should allow for the detection of the lung cancer (LC) and possibly at an earlier stage and thereby increase the effectiveness of clinical interventions. Here, our primary objective was to evaluate the potential of spontaneous sputum as a source of non-invasive metabolomic biomarkers for LC status.
Materials and methods: Spontaneous sputum was collected and processed from 34 patients with suspected LC, alongside 33 healthy controls. Of the 34 patients, 23 were subsequently diagnosed with LC (LC^+, 16 NSCLC, six SCLC, and one radiological diagnosis), at various stages of disease progression. The 67 samples were analysed using flow infusion electrospray ion mass spectrometry (FIE-MS) and gas-chromatography mass spectrometry (GC–MS).
Results: Principal component analysis identified negative mode FIE-MS as having the main separating power between samples from healthy and LC. Discriminatory metabolites were identified using ANOVA and Random Forest. Indications of potential diagnostic accuracy involved the use of receiver operating characteristic/area under the curve (ROC/AUC) analyses. This approach identified metabolites changes that were only observed with LC. Metabolites with AUC values of greater than 0.8 which distinguished between LC^+/LC^- binary classifications where identified and included Ganglioside GM1 which has previously been linked to LC.
Conclusion: This study indicates that metabolomics based on sputum can yield metabolites that can be used as a diagnostic and/or discriminator tool. These could aid clinical intervention and targeted diagnosis of LC within an 'at risk' LC^- population group. The use of sputum as a non-invasive source of metabolite biomarkers may aid in the development of an at-risk population screening programme for lung cancer or enhanced clinical diagnostic pathways.

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1. Introduction

Lung cancer (LC) is the most prevalent cancer in the world; responsible for 1.3 million deaths annually [1]. The last 30 years has seen little improvement in the overall five year survival rate for LC; with only 15% of patients living for at least five years after their initial diagnosis [2]. These relatively poor survival rates are primarily a result of the late detection of a malignancy; reducing the success of clinical interventions. Clinicians currently rely on three main tools for LC diagnosis: X-ray, computerised tomography (CT) scans, and bronchoscopy. These methods have improved our ability to detect lung cancer, but have nevertheless failed to improve the rate of early LC detection [3]. Another aspect of this poor early detection is the association of LC with smoking, which masks some of the disease's early symptoms, which has been linked to approximately 90% of LC tumours [4].

An alternative screening methodology to radiography, which is currently the most widely used approach, is the utilisation of molecular markers, both genetic and metabolomic, in biofluids. For example, microRNAs have been suggested as biomarkers for NSCLC in sputum [5], plasma [6], and serum [7]. Previous work by members of this research group has demonstrated that chemometric analysis combined with Fourier transform infrared spectroscopy is a non-invasive approach that allows for the discrimination of LC positive patients. This demonstrated that sputum could be used as a non-invasive source of biomarkers for LC [8]. However, analysis of mid-IR spectra only provides information on broad changes in classes of chemicals, and has a poor ability to resolve changes

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http://dx.doi.org/10.1016/j.lungcan.2016.02.006
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to particular chemicals. By comparison, metabolite profiling based on sample screening using Mass Spectrometry (MS) can resolve changes in individual chemicals and thus, could more readily identify biomarkers linked to LC detection.

The aim of this study was to employ MS metabolomic profiling to identify clinically relevant biomarkers in sputum that could be used for detect LC (diagnosis) as well as provide some pathophysiological insights based on the characteristics of the chemical biomarkers. We utilised two MS approaches in this study, Gas Chromatography MS (GC–MS) and Flow Infusion Electrospray MS (FIE-MS). Our rationale for this approach is that both MS technologies are widely used in biomarker discovery, but have differing levels of sensitivities and different approaches in regards to sample preparation and analysis. For example, GC–MS requires chemical derivatization of sample metabolites prior to analysis whilst FIE-MS requires no pre-treatment [9]. Although, our study employed both univariate and multivariate approaches our study sought to conform to the demands of the TRIPOD (The Transparent Reporting of a multivariable prediction model for Individual Prognosis Or Diagnosis) Statement by adhering to the recommended checklist [10]. We employed assessments of diagnostic accuracy based on receiver operating characteristic (ROC)/Area under the Curve (AUC) that suggest that our approach could be used in clinical context to inform the detection of the disease. To the best of our knowledge, metabolomic profiles have not been reported using sputum as a biofluid from clinical patients. Thus, beyond, the detection of biomarkers, a description of the LC sputum metabolome offers a novel insight into the pathology of LC.

2. Materials and methods

2.1. Ethics statement

The MedLung observational study (UKCRN ID 4682) received loco-regional ethical approval from the Hywel Dda Health Board (05/WMW01/75). Written informed consent was obtained from all participants at least 24 h before sampling, at a previous clinical appointment, and all data was link anonymised before analysis.

2.2. Study design

This study aimed to compare the metabolomes of three groups of sputum samples. Two sets of sputum samples were obtained from patients referred to the access LC clinic at the Prince Phillip Hospital, Wales, UK; a site of primary care. Lung cancer status was subsequently assessed as part of the Medlung observational study (UKCRN ID 4682) and patients were classified as either LC^+ or diagnosed with another pulmonary disease (LC^-) based on histological assessments of sputum bronchoscopy derived samples (Table S1). Metadata including comorbidities, smoking history and drug history are given in Table S1. Additionally, spontaneous sputum samples were collected from staff members at Swansea University who had no previous history of cancer or lung disease, other than asthma. These non-clinical samples were designated as a control (CON) group. The design expensively exploited pairwise analyses between LC^+ and LC^- groups and the CON group. As this project was seen as a pilot project, no external validation set, comprising, for example, testing on another set of patients samples was used. Further, the danger of over-fitting the derived data was reduced through the extensive use of simple two-way ANOVA in our pairwise comparisons. Sampling occurred between 2012 and 2013 to align with the MedLung study timeline and this, rather than an *a priori* design target, governed the number of samples analysed.

2.3. Patient recruitment and sampling

Spontaneous sputum was collected from referrals to our rapid access LC clinic at the Prince Phillip Hospital, Wales, UK or volunteers from staff members at Swansea University. No *a priori* criteria were applied to the selection of patients or volunteers other than their ability to produce sputum. Patients were asked to cough into sterile, 50 mL polypropylene tubes (Greiner Bio-One Ltd., UK) prior to bronchoscopy, to at total volume of 2–3 mL. A 100 μL aliquot of all samples, including the CON group, was taken to create a second pellet that was subsequently formalin fixed and wax embedded prior to sectioning and staining with haemotoxylin and eosin (H&E). To confirm samples were of bronchial origin, H&E stained sections were assessed by a consultant histopathologist for presence of bronchial epithelial cells. Histological assessments of the LC^+ class allowed the recording of LC type and stage. Thus, NSCLC classifications were obtained for sixteen samples and six were SCLC. Only in one case (LC06) was no classification obtained. Within the NSCLC samples, seven could be sub-classified as adenocarcinoma type and five squamous cell types. Considering the LC^- classified samples, three were diagnosed with chronic obstructive pulmonary disease (COPD) and two with pneumonia. Amongst the LC^+ group, only two (LC07, LC20) were diagnosed with COPD which could be considered a LC co-morbidity and none with pneumonia.

2.4. Processing of raw sputum

In line Raw sputum samples were frozen at −80 °C and defrosted in ice for approximately two hours when required. Sputum cells were isolated by adding 0.5 mL of a working solution of dithiothreitol (DTT), made up by adding 2.5 g of DTT to 31 mL of 30% aqueous methanol, and 5 mL of 30% aqueous methanol. The samples were then placed on a vortex mixer for 15 min and underwent centrifugation at 1800*g* for 10 min. The supernatant was removed and the pellet used in subsequent metabolomic profiling.

2.5. Flow infusion electrospray mass spectrometry (FIE-MS)

After processing, 20 μL of the sputum pellet was added to 20 μL of ultrapure water and 40 μL of ice-cold HPLC grade acetone. Samples were vortex mixed for five seconds, cooled on ice for 30 min, and then underwent centrifugation at 11,000*g* for five minutes. After centrifugation, 50 μL of the supernatant was removed and 250 μL of 70% methanol (made up using HPLC grade methanol and ultrapure water) was added. Glass vials were capped and analysed in random order on a LTQ linear ion trap (Thermo Electron Corporation). Data were acquired in alternating positive and negative ionization modes over 4 scan ranges (15–110, 100–220, 210–510, and 500–1200 *m*/*z*), with an acquisition time of five minutes. The resulting mass spectrum was the mean of 20 scans about the apex of the infusion profile.

2.6. Gas chromatography mass spectrometry (GC–MS)

The sputum pellet was processed as described in Section 2.4 and 50 μL of the supernatant after centrifugation removed and dried using a DNA SpeedVac (Savant, USA) at 40 °C. After removal of all liquid, 30 μL of a 20 mg/ml solution of methoxyamine dissolved in pyridine was added and each sample was transferred to a 11 mm diameter glass GC vials which were capped with Teflon crimp caps and incubated at 90 °C for 15 min. After cap removal, 20 μL of *N*,*O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was added to the sample, alongside 5 μL of an alkane standard mix. This mixture comprised of C_{10}, C_{13}, C_{15}, C_{18}, C_{19}, C_{23}, C_{28}, C_{32} and C_{36} alkanes dissolved in pyridine each at a concentration of 2 μL/mL (for alkanes liquid at room temperature) or 2 mg/mL (for alkanes solid at room

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