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Research paper

Non-invasive exploration of metabolic profile of lung cancer with Magnetic Resonance Spectroscopy and Mass Spectrometry

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ABSTRACT

Background: Lung cancer is a major cause of global morbidity and mortality. Current low dose CT screening is invasive and its role remains contentious. There are no known biomarkers to monitor treatment response, detect disease recurrence and patient selection for adjuvant treatment after curative surgical resection. Hence there is an urgent need to explore non-conventional and non-invasive tools to develop novel biomarkers to improve the outcome of this lethal cancer.

Methods: This is an ongoing exploratory and translational study involving collection of bio fluids from 50 patients with early stage non-small cell lung cancer before and after surgical resection. The primary objective is to identify cancer specific metabolome in body fluids - sputum, exhaled breath condensate, blood and urine of the patients with early stage non-small cell lung cancer using Magnetic Resonance Spectroscopy and Mass Spectroscopy.

Conclusion: The trajectory of change in metabolic profile of body fluids before and after surgical resection may have potential clinical applications in lung cancer screening, as biomarkers for disease recurrence and exploration of novel targets for therapeutic intervention.

1. Introduction

There has been major advancement in our understanding of molecular biology of lung cancer within the last 15 years. This research has led to innovative targeted drug therapy in lung cancer with optimism and improved outcome for patients with new diagnoses of lung cancer [1].

However, the issue of lung cancer screening to detect early stage lung cancer remains contentious, not the least of which is due to the invasiveness and cost of current screening methods [2]. Unfortunately, most patients present with advanced stage disease when treatment may or may not be possible [3]. In those who receive local or systemic treatment, there is absence of biomarkers to monitor their response to treatment and for disease recurrence. Biomarkers to monitor treatment response and recurrence status in a non-invasive fashion would represent significant improvement in the delivery of care. Current trends in modern precision medicine indicate an essential need to explore novel biomarkers [4]. In the current study, we are exploring the metabolic profile of patients with lung cancer before and after surgical resection using MRS and MS. This may lead to identification of metabolic profiles that can potentially serve as the aforementioned biomarkers.

2. Scientific rationale

Normal body cells transform their biologic and metabolic behavior

Abbreviations: **MRS**, magnetic resonance spectroscopy; **NMR**, nuclear magnetic resonance; **MS**, mass spectrometry; **LC**, liquid chromatography; **LC-QTOF-MS**, liquid chromatography quadrupole time-of-flight mass spectrometry; **EBC**, exhaled breath condensate; **NSCLC**, non-small cell lung cancer

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and microenvironment after acquiring genetic alterations; namely activation of certain oncogenes and loss of certain tumor suppressor genes. This leads to alteration of cellular signaling pathways, PH, nutritional status and oxygen status of the cells. Normal existing mitochondrial oxidative phosphorylation is replaced by the newly activated glycolytic pathway as a source of nutrition to the rapidly proliferating and demanding malignant cells. Metabolic changes accompanied by oncogenes and proteins such as HIF-1a., RAS, C-MYC, SRC, and p53 result in tumorigenesis and metastasis [5–12].

MRS and MS are two powerful tools to study metabolic changes at the cellular level. MRS is essentially a somatic expression of nuclear spins at atomic levels influenced by number of protons and neutrons in the nuclei, the concentration of nuclei in a given organic substance and the applied external magnetic field. With MRS there is minimal sample preparation and rapid detection of multiple metabolites in a single experiment [13,14]. MS on the other hand is 100 times more sensitive than MRS and can detect the metabolites with low concentration. MS analysis of biological samples is typically performed with LC [14].

Our team has recently completed and published a feasibility study on bio-fluids obtained from advanced stage lung cancer patients using MRS. We have demonstrated absence of glucose in the sputum and decreased concentration of methanol in breath condensate of pathologically proven NSCLC in comparison to patients without lung cancer [15].

Our current study is unique to explicitly enroll early stage lung cancer patients who have surgically resectable cancer. Bio-fluids from the enrolled patients will be obtained before and surgical resection; hence providing the control arm of the study from same group of patients

We hypothesize that *MRS and MS are powerful metabolomics tools for in vitro diagnostics and can yield a specific metabolic phenotype of lung cancer obtained from sputum, EBC, blood and urine of early stage lung cancer patients.*

3. Methods

3.1. Ethics

The study is conducted in accordance with the declaration of Helsinki. Written approvals were obtained from the Research Ethics Boards at the University of Manitoba (H2017:247), Research Impact Committees of Health Science Center, Cancer Care Manitoba, Diagnostic Service Manitoba and St Boniface General Hospital prior to implementation of the study and enrollment of any subject. The tenets of Good Clinical Practice have been followed in the completion of this study. Eligible patients will receive written and oral information about the goals and methodology of the study from a qualified research assistant and will be asked for informed consent. Subjects are free to withdraw from the study at any time.

3.2. Patient selection and enrolment

Fifty patients with early stage NSCLC who are candidates for surgical resection will be enrolled over two years. Patients will be screened at thoracic surgical clinic. Patient's clinical characteristics, CT/PET images and pathology will be reviewed by the principal investigator or one of the clinical co-investigators. If deemed eligible, patients will be contacted and introduced to the study. A dedicated and formally trained research assistant will obtain informed consent from each patient and collect bio-fluid specimens before and after surgical resection as described in Fig. 1.

3.3. Specimen collection

A set of samples from the patients consisting of sputum, EBC, blood and urine will be collected within and up to 4 weeks before surgical resection and then another set of samples of the same bio-fluids will be collected within and up to 4 months after the surgical resection before any further therapeutic interventions including chemotherapy, immunotherapy, targeted therapy or radiation.

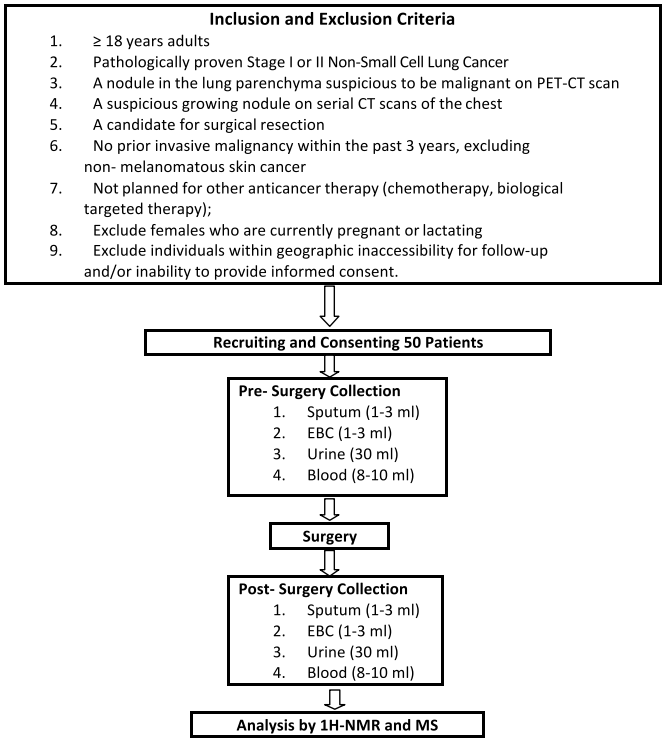

Fig. 1. Schema of Procedures.

3.3.1. Exhaled breath condensate (EBC) collection

Approximately 1–3 ml of EBC will be obtained by having the participant exhale into a cooled collecting container (a custom-built condenser using a metal element that surrounds a tightly fit inert collection tube) while breathing comfortably through a mouthpiece with a nose clip in place for 20–30 min according to our previously published protocol [15] (Fig. 2).

3.3.2. Sputum collection

Induced sputum samples from the inhalation of 4 ml of hypertonic saline solution will be collected. Sputum would be confirmed cytologically by the presence or absence of macrophages by a dedicated pathologist as in our feasibility study [15]. Some patients may yield sputum spontaneously and may not require induction.

3.3.3. Serum collection

About 8–10 ml of blood sample will be collected into a sterile vacutainer tube. No additives such as EDTA, citrate, Li-heparin and other

Fig. 2. EBC collection.

added stabilizers will be used; as they may give additional signals in the NMR spectra. Similarly no gel containing tubes will be used as they separate blood cells from serum. Collected blood sample will be left at room temperature for 30–40 min to coagulate and spun for 15 min; followed by centrifugation at 4000 RMP, 4 °C for 15 min. The resulting serum will be collected into cryo vials.

3.3.4. Urine collection

Mid-stream urine will be collected into a sterile cup.

All 4 types of samples will be collected and stored at −80 °C until MRS and MS experiments are performed.

3.4. Nuclear Magnetic Resonance (NMR) data collection and analysis[1]

Frozen EBC, sputum, serum and urine samples will be thawed on ice for 20–30 min. 500 µl of neat EBC will be transferred into an Eppendorf tube, to which 75 µl of D_2O and 25 µl of Trimethylsilylpropanoic acid (TSP),(0.75%) solution will be added. 300 µl of sputum will be transferred into a tube, to which 300 µl of 2 M NaCl solution (pH = 7.4) and 20 µl of TSP, > 0.75%) will be added. A volume of 300 µl of phosphate buffer (pH = 7.4) containing 5 µM NaN_3 will be added to each serum sample in 1:1 ratio (v/v), and then 20 µl of TSP (0.75%) will be added. For urine analysis, 400 µl of sample will be mixed with 230 µl of 0.2 M phosphate buffer (0.2% NaN_3) solution and 70 µl of Chenomx ISTD (IS-2, 5 mM DSS, 0.2% w/v NaN3) solution. Samples will be mixed by vortex and centrifuged at 12,000 rcf, 4 °C for 5 min. A volume of 600 µl of each prepared sample will be transferred into a 5 mm NMR tube for analysis.

The NMR experiments will be conducted on a Bruker Ascend 600 Spectrometer, operating at 600.27 MHz for proton nuclei and 150.938 MHz for carbon nuclei. Each sample will be analyzed using NMR within 24 hrs of being prepared, with a probe temperature of 298 K. Two different scans will be run for each sample with a few randomly selected samples running Heteronuclear Single Quantum Coherence (HSQC) for additional analysis. The proton will be run with a 65.5 k time domain, a 90° pulse width of 10 µs, a spectral width of 16 ppm, and a relaxation delay of 5s. The number of scans is 32 with 2 dummy scans producing an acquisition time of 4.75 min. The Nuclear Overhauser Effect Spectroscopy (NOESY) suppresses the water at 2819 Hz, with a time domain of 32.7 k, a 90° pulse width of 10 µs, a spectral width of 16 ppm, and a relaxation delay of 5s. The 7.75 min acquisition time is produced by 64 scans and 4 dummy scans. The 2D NMR will have a 90° pulse width of 10µs and a relaxation delay of 1.5s. The time domain values for F1 and F2 will be 2048 and 400, respectively. The spectral width will be set to 16 ppm (F1) and 210 ppm (F2), the acquisition time will be approximately 11 h to cover32 scans and 16 dummy scans. The spectra will be processed using MestReNova version 12.0.0–20080. The metabolomics analyses will be performed using Chenomx NMR suite 8.2 professional.

3.5. Liquid chromatography quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) data collection and analysis

All frozen samples will be thawed on ice for 20–30 min. An aliquot (250 µl of urine) will be transferred into an Eppendorf tube, to which 10 µl of norvaline solution (0.03 mg/ml) and 500 µl of Acetonitrile (ACN) will be added. The mixture will be left at −20 °C for 30 min (quenching step). 100 µl of sputum will be added to 100 µl of H_2O, and vortexed and placed at −20 °C and freeze-dried under 0.016 mbar at −53 °C. Dried sputum samples will be mixed with 20 µl of standard solutions (15 mg/ml), 150 µl of MeOH and 150 µl of ACN, and will be sonicated for 5 min. Serum aliquots (100 µl) will be mixed with 20 µl of internal standards solution (15 mg/ml) and 200 µl of ACN. EBC (110 µl) will be mixed with 10 µl of internal standard solution (Norvaline, 0.03 mg/ml) and 30 µl of ACN and centrifuged at 12,000 rcf at 4 °C for 20 min.

Processed EBC sample will be directly transferred into a glass insert in a GC vial for LC-QTOF- MS analysis. Supernatants of sputum, serum and urine will be transferred into new tubes and dried under nitrogen flow and stored at −20 °C prior to use. Dried serum and sputum samples will be reconstituted with 100 µl of ddH_2O: ACN (1:4) and dried urine sample will be constituted with 200 µl of ddH_2O: ACN (4:1). Each reconstituted sample will be transferred into a glass insert in a GC vial for MS analysis as previously described [16–18].

Data acquisition will be performed on a 1260 Infinity HPLC coupled to a 6538 UHD Accurate Q-TOF MS system (Agilent Technologies, CA, USA) equipped with an electrospray ionization (ESI+) source.

Urinary, EBC and sputum metabolites will be separated on a 2.1 mm × 100 mm, 1.8 µm Zorbax SB-Aq column (Agilent Technologies) with the column temperature maintained at 45 °C while serum metabolites will be separated on a 2.1 mm × 50 mm, 1.8 µm Zorbax Extended-C18 column (Agilent Technologies) with the column temperature maintained at 60 °C. Mobile phase A (ddH_2O) and B (Acetonitrile) with 0.1% formic acid will be used for both methods. The flow rate will be maintained at 0.4 and 0.5 mL/min for urine and serum metabolites analyses, respectively.

The gradient program for urine, EBC, sputum and serum samples will be as follows: 0 (2%) to 13 min (95%) of solvent B for urine, EBC and sputum samplesand 0, 0.5 and 16 min with 30%, 30% and 100% of solvent B, respectively, for serum samples. There will be a post-run time of 2 min with buffer (TFANH4, Hp, purine) before the next sample injection. The auto-sampler is maintained at a temperature of 5 °C.Gas temperature, drying N2 gas flow rate and nebulizer pressure for MS will be maintained at 300 °C, 11 L/min, and 45 psig. Capillary voltage and fragmentor voltage are 4000 V and 175 V, respectively. Mass detection will be operated by using electrospray with reference ions of m/z 121.050873 and 922.009798 for both of positive and negative mode. A full range mass scan from 50 to 1700 m/z will be used, and the data acquisition rate is maintained at 2 spectra/s. When detected and separated in LC-MS, raw data files will be acquired and stored as "*.d" files using Agilent MassHunter Acquisition software (B.07).

Raw "*.d" files will be processed in Agilent MassHunter Qualitative (MHQ B.07) where the Molecular Feature Extraction will be used as the first algorithm to extract the detected compounds from the "*.d" files. The parameters will be set to allow the detection and extraction of features satisfying absolute abundances of more than 3000 counts and to provide information regarding $[M+H]^+$, isomers and their corresponding $NH4^+$, Na^+, and K^+ adducts. Potential formulas will be generated for the extracted compounds by the Generate Formulas algorithm using collected information such as retention time, exact masses and abundances. After converting into compound exchange format (*.cef) files by the Export to CEF algorithm, extracted features is ready for further comparative and statistical analyses by Mass Profiler Professional (MPP, 12.6) [16–18].

3.6. Statistical analysis

3.6.1. Sample size

The sample size (n = 50) should be sufficient to detect systematic changes in several biomarkers. All the subjects have lung cancer; there is no gold standard. The study will not deal with diagnostics, specificities or sensitivities. Further it should be noted that the 2 groups for comparing metabolites are from the same patient population before and after surgical resection of the pathologically proven lung cancer.

3.6.2. Analysis of the detected metabolites

Fold change analysis using paired T-tests ($P < 0.05$; ≥ 2-fold changes) with an asymptotic P value computation and a multiple-

[1] Magnetic Resonance Spectroscopy (MRS) and Nuclear Magnetic Resonance (NMR) are used interchangeably.

Table 1
Patients enrolled and biofluids collected.

	Urine	Serum	EBC	Sputum	Cytological confirmed sputum
Pre-surgery	50	50	50	50	32
Post-surgery	37	36	37	37	22
Both pre and post surgery samples	37	34	37	36	17

testing correction (Benjamini-Hochberg) will be applied to examine statistical significance of metabolites detected in EBC, sputum, serum and urine samples using the MPP software (12.6). Identification of those compounds will be confirmed by mass spectra, retention time and confidence scores against the Metlin database (>79,000 metabolites, 39,000 lipids and 168,000 peptides). Biochemical pathways of selected metabolites will be analyzed using the KEGG (Kyoto Encyclopedia of Genes and Genomes) database and the architect pathway (MPP, 12.6).

3.6.3. Clinical correlation

Metabolic profile obtained from the samples with NMR and MS will then be analyzed for key metabolic markers and correlated with the clinical, pathological and radiological characteristics of the patients in addition to pre surgical Standard Uptake Value (SUV) on Positron Emission Tomography (PET) and molecular mutations including EGFR, ALK, PDLI. Data will be analyzed using a paired *t*-test (if assumptions of the test are met) to report the mean changes in the concentrations of metabolites before and after surgical resection and the standard deviations of changes among the participants. Similar, but less powerful, non-parametric tests will be applied if the assumptions necessary for the paired t-test are violated. Correlation analyses will be used to examine the associations between concentrations of metabolites before and after the surgical resection. Regression analyses will examine relationships of metabolite levels and level changes with clinical and pathological variables of the patients.

4. Current study progress

Patient enrolment began in Feb. 2018. To date, we have successfully enrolled 50 patients as in Table 1. All the collected sputum specimens have been further processed for cytological evaluation.We hope to complete data collection and data analysis within the next 3months. Post-operative sample collection is continued.

5. Clinical significance and future directions

The current study will provide an opportunity to study the metabolic changes in relation to clinical, radiologic and pathological features of early stage lung cancer when there is minimal gross disease. Hence, bio-fluid specimens taken before and after surgery (i.e. to remove the lung cancer) will provide a direct comparison of metabolic changes in the same patient population with and without lung cancer. The results of our study may open new avenues for lung cancer screening, new biomarkers for patient selection for adjuvant therapy post-resection, detect subclinical recurrence and identification of metabolic targets for new drug therapy.

Conflicts of interest

The authors declare that they have no conflict of interest.

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