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Metabolic Profiling of Human Lung Cancer Blood Plasma Using 1H NMR Spectroscopy

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Abstract. Lung cancer (both small cell and non-small cell) is the second most common cancer in both men and women. The article represents results of evaluating of the plasma metabolic profiles of 100 lung cancer patients and 100 controls to investigate significant metabolites using 400 MHz 1H NMR spectrometer. The results of multivariate statistical analysis show that a medium-field NMR spectrometer can obtain the data which are already sufficient for clinical metabolomics.

INTRODUCTION

Lung cancer was the most commonly diagnosed cancer as well as the leading cause of cancer death in males in 2008 globally. This type of cancer accounts for 13% (1.6 million) of the total cases and 18% (1.4 million) of the deaths in 2008 [1]. The known risk factors for lung cancer include exposure to carcinogens from air pollution, tobacco smoke, exposure to asbestos and deep-fried food [2, 3, 4]. Therefore, an investigation of lung cancer is an important part of cancer research. Metabolomics is suitable to study lung cancer because a metabolomic profile accumulates knowledge on genome, transcriptome and proteome [5]. The nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) are two main techniques used in the metabolomics. The latter is often combined with gas chromatography (GC) or high-performance liquid chromatography (HPLC). The advantages of using NMR for metabolomic studies are fastness, robustness and non-destructiveness. NMR is a suitable method for analysis of biofluids such as urine, serum or blood plasma, saliva, etc. For instance, X. Zhang e*t al.* investigated the ability of 1H NMR-based metabolomic approach to identify metabolomic changes at stage I of lung cancer [6]. Increased levels of lactate, ketone bodies and several amino acids (including glutamate, glutamine, histidine, and tyrosine) were detected. Decreased levels of glucose (α- and β-glucose), lipids, unsaturated lipids, phospholipids intermediates (choline, phosphocholine, and glycerophosphocholine), trimethylamine N-oxide (TMAO), and betaine were observed in lung cancer patients compared to healthy controls. Another study [7] is demonstrated lower levels of lipoproteins, glutamine, threonine and histidine; higher serum levels of leucine/isoleucine, N-acetyl-cysteine, glutamate, and creatine. However, despite all advantages 1H NMR-based metabolomic studies of lung cancer are still rare [8, 9, 10]. In our opinion, one of the limiting factors for spreading of 1H NMR-based metabolomic studies is high cost and housing of 600 MHz NMR spectrometer, which is commonly used for those type of research, while 400 MHz instruments are more spread and common. Therefore, in the present study, we focused on the demonstration of the ability of applying 400 MHz 1H NMR spectrometer for plasma metabolic profiling of metabolic biomarkers associated with lung cancer.

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EXPERIMENTAL PART

Chemicals and materials

K_2HPO_4, NaN_3 and TSP-2,2,3,3-d_4 were purchased from Sigma-Aldrich. ^2H_2O was purchased from Cortecnet. NMR tubes were purchased from Bruker Biospin Ltd. (Germany).

Sample collection

The procedures followed in this study were performed in accordance with the Helsinki Declaration (1964, amended in 1975 and 1983). This study was approved by the institutional review board of Tomsk Cancer Research Institute, Tomsk, Russia (#13b, 2014 September, 27) and all patients signed an informed consent for voluntary participation. Venous blood samples were collected into EDTA spray-coated tubes and fractionated into plasma and blood cells within 4 hours after sampling. Blood was centrifuged for 20 min at 400 × g, then transferred into a new tube and centrifuged for the second time for 20 min at 1.200 × g. Supernatants were stored frozen in aliquots at −80 °C.

Plasma sample preparation and NMR data acquisition

Plasma sample preparation and NMR data acquisition were produced according to the protocol of A. Verhoeven with some minor modifications [11]. Prior to measurements, plasma samples were thawed at 4 °C, then 350 µL of plasma from each sample were mixed with 350 µL buffer solution in ^2H_2O (pH = 7.4) containing 0.142 M of disodium phosphate buffer, 2 mM of NaN_3 and 4 mM of TSP-2,2,3,3-d_4 as an internal standard and chemical shift reference (0.4 mM final concentration in each sample). Finally, 600 µL of each plasma-buffer mixture were transferred to 5 mm NMR tubes and placed in refrigerated racks (6 °C) of a SampleJet system prior to the NMR measurements.

The NMR data were recorded using a Bruker 14.1T AVANCE II spectrometer for ^1H 400 MHz. Each sample was allowed to be situated in the probe for 5 min to adopt a stable temperature at 27 °C before starting the calibration routines and data acquisition. The probe was then automatically tuned and matched, followed by shimming and proton pulse calibration. For each sample, three 1H NMR spectra were acquired with water peak suppression: a standard nuclear Overhauser effect spectroscopy pulse sequence (noesygppr1d in Topspin 3.0 library); a Carr–Purcell–Meiboom–Gill (cpmgpr1d.comp in Topspin 3.0 library) spin-echo sequence to suppress signals arising from molecules with a high molecular weight; and a diffusion edited sequence (ledbpgppr2s1d.comp in Topspin 3.0 library) with a diffusion time of 120 ms. The spectra were automatically phased and baseline corrected and referenced to the internal standard chemical shift (TSP; δ 0.0 ppm). An evaluation of spectra quality was performed after processing. Peaks' line-width was evaluated with the TSP singlet and methyl protons doublet of alanine. In addition, the efficiency of water suppression and the quality of the baseline were also checked. The spectra that failed to fulfil the quality criteria were discarded from further analysis. Two-dimensional J-resolved spectra (2D *J*res) were also collected for each sample using the same water suppression scheme as described above during the relaxation delay of 2 s. The FIDs (Free induction decays) were automatically processed with Fourier transformation, and spectra were referenced to the TSP signal at 0.0 ppm in the F2 dimension and at 0.0 Hz in the F1 dimension.

For the purpose of assignment, 2D NMR spectra were also acquired for a sample made as amixture of all plasma samples. The set of 2D experiments included ^1H-^1H correlation spectroscopy (COSY);^1H-^1H total correlation spectroscopy (TOCSY);^1H-^{13}C heteronuclear single quantum correlation (HSQC); and ^1H-^{13}C heteronuclear multiple bond correlation spectroscopy (HMBC) using the standard parameters implemented in Topspin 3.0 library (Bruker Biospin Ltd.).

Data processing and statistical analysis

The NOESY spectra were used for statistical analysis. Preprocessing of NMR data to be suitable for statistical analysis was performed with in-house routines written in Matlab 2014a (The Mathworks, Inc., USA) and Python 2.7 (Python Software Foundation, www.python.org). All 1D NMR ^1H spectra were re-evaluated for incorrect baselines and corrected using a polynomial fit of degree 5. The spectral region from 0.5 to 9.5 ppm was binned using an in-house algorithm for adaptive intelligent binning [12]. The initial bin width was set to 0.02 ppm and final variable bin sizes were calculated based on the peaks' edges in the spectra by using a lowest standard deviation criterion. The spectral region including the residual water was excluded from the data.

The final data consisted of 393 bins of variable size, which were normalized by the Probabilistic Quotients

Normalization method (PQN) [13] to correct for dilution differences from sample to sample. Finally, the normalized data was scaled to unit variance for the statistical analysis.

Analysis of the final data was performed with principal component analysis (PCA) projection to latent structure discriminant analysis (PLS-DA) by using SIMCA 14 software package (Umetrics, Sweden) [14]. IBM SPSS Statistics 23 (IBM corp., USA) was employed for independent samples T-test (Confidence interval = 95%).

RESULTS AND DISCUSSION

Characteristics of the study participants

The study group consisted of patients with lung cancer in the age range of 39-70, and the age range of the control group was from 40 to 70. Both groups had an identical percentage of men and women, where men were the vast majority. More than 50% of cancer patients had a III or IV stage of lung cancer and 72% had non-small-cell lung cancer (NSCLC); 14% had small-cell lung cancer (SCLC). All participants were smokers except one patient in the controls and one patient in the case group, which led to complexity of recruiting healthy patients, thereby, in the study patients, whom had bronchitis and another lung diseases were included. Characteristics of patients are presented in Table 1.

TABLE 1. Characteristics of the study participants.

Parameter	Cancer	Control
N		
total	100	100
male	88	73
female	12	27
Age, year		
mean	60.4	51.2
range	39-79	40-70
Smoking, years		
mean	36.7	25.5
range	0-60	0-53
Earlier diseases		
Bronchitis	31	39
Pneumonia	12	8
Stage		
I-II	24	-
III-IV	57	-
Unknown	19	-
Histological type		
NSCLC	72	-
SCLC	14	-
Unknown	14	-

1H NMR Spectroscopy of blood plasma

An example of 1H NMR spectra of a blood plasma sample obtained from a control healthy patient is shown in Fig.1. Identification of metabolites was performed by exhaustive search of the total 1D and 2D *Jres* data using the proprietary Bbiorefcode (Bruker Biospin Ltd.) database. The IDs of the annotated resonances were further verified by the collected 2D NMR data. The spectra were dominated by a number of metabolites, including lipid fractions (e.g., fatty acids, triacylglycerides and lipoproteins), valine, lactate, alanine, citrate, creatinine, glucose, amino acids and tyrosine.

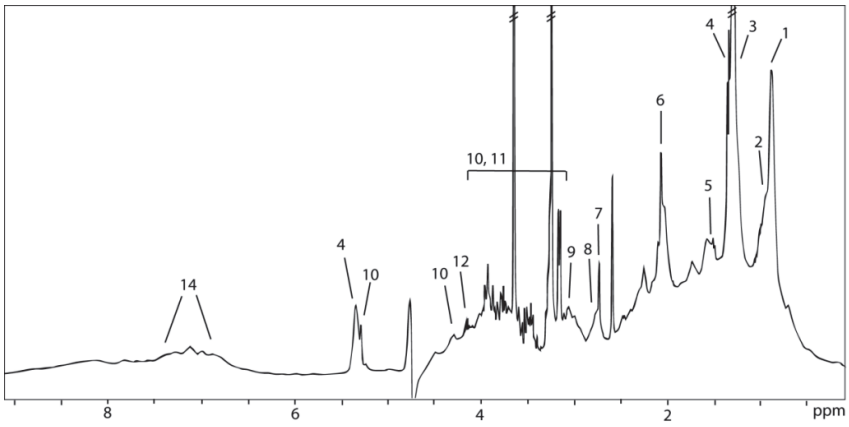
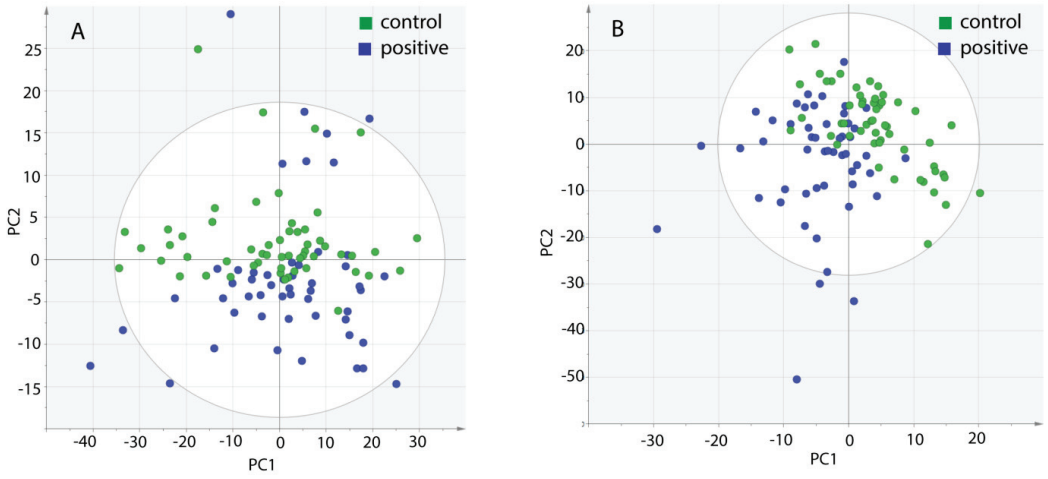


FIGURE 1. Typical 400 MHz 1H NMR NOESY spectra of blood plasma obtained from a control conditionally healthy patient. Keys to Fig.: 1, lipid CH_3; 2, valine; 3, lipid CH_2; 4, lactate; 5, alanine; 6, lipid =CH-CH_2-; 7, citrate; 8, lipid =CH-CH_2-CH=; 9, creatinine; 10, glucose; 11, amino acids CH; 12, lipid =CH-; 13, tyrosine.

PCA and PLS-DA of 1H NMR data

PCA was carried out on the normalized scaled binned the NOESY spectra dataset. The plot of the PCA scores from plasma of control and study groups of patients is shown in Fig. 2a. Twenty components were calculated and the model corresponding to the first three PCs explained 80.7% (PC1 = 51.4%; PC2 = 14.2%) of the total variance. Separation between the study group and the controls was achieved predominately in the second PC.



(a) (b)

FIGURE 2. PCA (a) and PLS-DA (b) scores from NOESY spectra of plasma from patients with lung cancer (blue) and control group of patients (green)

Having demonstrated the clustering related to the status of a patient by using PCA, nevertheless, we subsequently investigated the data by using PLS-DA to maximize the separation between the groups of objects (Fig. 2b). Judged from the cumulative values of R^2X (0.62), R^2Y (0.41), Q^2 (0.35), and p-value of CV-ANOVA ($6.1*10^{-8}$) we can consider that the model is statistically significant and can be valid for selecting variables. Based on the variable importance in the projection (VIP) value, the variables were chosen, annotated and presented in Table 2.

The changes in metabolites are presented in Fig. 3. Box-plots were created from data of the most significant resonances based on VIP value for each metabolite represented in Table 2.

TABLE 2. The dominant metabolites observed in plasma obtained from patients with lung cancer compared with healthy patients.

Metabolite	Chemical shift δ, ppm	Variable importance (VIP)
D-glucose	3.88	2.7
	3.85	2.65
	3.74	2.07
	3.7	1.7
Alanine	3.78	2.51
	1.5	2.1
Glycerol	3.54	2.42
	3.82	2.27
Glycoproteins	2.04	1.62
Lipids (mainly LDL)	0.83	1.45
	1.31	1.36
	1.29	1.22
Lipids (mainly LVDL)	0.88	1.36

The dominant metabolites are connected with energy, amino acid and lipid metabolism, which is conformed with other studies [6, 7, 15, 16]. According to the PLS-DA model, the most dominant metabolite is glucose, which is noted at a higher level in plasma of patients with lung cancer as compared to controls. The same effect was observed in other studies [16, 17], an observation that might be explained by an increase of gluconeogenesis in cancer cells, where an excess of glucose is utilized in the blood stream [18, 19, 20]. The level of alanine significantly decreased in plasma of cancer patients as relative to healthy controls, which comports with other plasma metabolomic study of lung cancer [21]. Other metabolites are a part of lipid metabolism: The levels of low-density lipoproteins (LDL) and very low-density lipoproteins (VLDL) in plasma of patients with tumors are lower in comparison with controls; glycoproteins and glycerol are higher in the plasma of the case group versus the one in the control group. This finding agrees with the hypothesis about the changes in membrane synthesis in cancer cells [22, 23, 24].

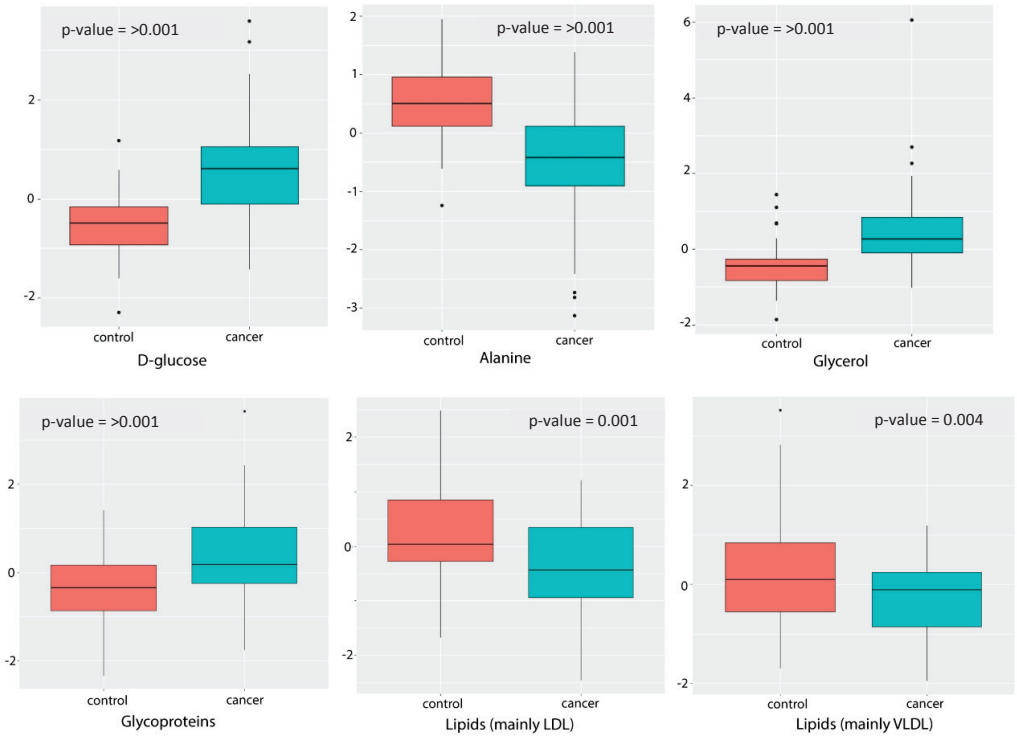


FIGURE 3. Box-plots of the dominant metabolites observed in plasma obtained from patients with lung cancer compared with healthy patients

CONCLUSIONS

In conclusion, it can be said that a medium-field (400 MHz) NMR spectrometers can be sufficient for metabolomic case-control studies of lung cancer. The present research shows that the significant metabolites are connected with energy, amino acid and lipid metabolism, which is conformed with previous studies which were provided by using more strongly field-magnet spectrometers (600 MHz).

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