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Diagnosis of lung tumor types based on metabolomic profiles in lymph node aspirates

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Abstract

Background: Treatment of lung cancer is evolving from the use of cytotoxic drugs to drugs that interrupt pathways specific to a malignancy. The field of metabolomics has promise with respect to identification of tumor-specific processes and therapeutic targets, but to date has yielded inconsistent data in patients with lung cancer. Lymph nodes are often aspirated in the process of evaluating lung cancer, as malignant cells in lymph nodes are used for diagnosis and staging. We hypothesized that fluids from lymph node aspirates contains tumor-specific metabolites and are a suitable source for defining the metabolomic phenotype of lung cancers.

Patients and materials: Metabolic profiles were generated from nodal aspirates of ten patients with adenocarcinoma, ten with squamous cell carcinoma, and ten with non-malignant conditions using time-of-flight mass spectrometry. In addition, concentrations of selected metabolites participating in the kynurenine and glutathione pathways were measured in a second set of aspirates using tandem mass spectrometry.

Results: A list of consensus features that separated these three groups was identified. Two of the consensus features were tentatively identified as kynurenine and as oxidized glutathione. It was shown that metabolite concentrations in these pathways are different for patients with and without malignancy.

Conclusion: Together the data suggest that metabolomic analysis of lymph node aspirates can identify tumor-specific differences in cancer metabolism and reveal novel therapeutic targets. This proof-of-concept study demonstrates the validity to complement and refine diagnosis of lung cancer based on metabolic signature in lymph node aspirates.

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Micro abstract: Treatment of lung cancer is evolving from the use of cytotoxic drugs to drugs that interrupt metabolic pathways specific to a malignancy. We report here in that the metabolic phenotype of lung cancer can be determined in lymph node aspirates harboring malignant tumor cells. Knowledge about metabolic activity of malignant tumor cells may aide to personalize therapy.

Introduction

Tumor biology as a basis for therapeutic intervention selection is transforming the diagnosis and treatment of lung cancer [1,2]. Most recently, therapies targeting tumor metabolism have shown promising results in pre-clinical studies [3–5]. Several investigators have studied metabolic fingerprints in serum and urine of patients with lung cancer [6], but most reported alterations have been variable or even discrepant, thus preventing identification of unique, robust, and consistent patterns [6]. These inconsistencies are probably caused by the fact that lung cancer cells contribute only minimally to the systemic metabolic profiles obtained from serum and urine.

Lymph node aspirates are frequently obtained in the process of diagnosing and staging lung cancer. Diagnoses are based on malignant cells in the lymph node aspirates (Fig. 1). The fact that there is intimate contact of malignant cells with aspirate fluid led us to hypothesize that lymph node aspirates are a suitable source material for defining the metabolic phenotype of malignant tumor cells, overcoming the shortcomings cited above. We sought first to define metabolite features in lymph node fluid characteristic for each of three categories; adenocarcinoma (AdenoCa), squamous cell carcinoma (SqCCa), and aspirates cytologically free of malignancy (NM). The biological relevance of the identified features was then evaluated by taking selected features, identifying them, and demonstrating important difference in corresponding metabolic pathways between aspirates containing malignant cells and aspirates free of malignancy. The goal of these two studies was to document the validity and utility of metabolite profiling of lymph node aspirates obtained in the process of clinical diagnosis and management of lung cancer.

Methods

Human subjects

The University of Arkansas for Medical Sciences Institutional Review Board approved the study protocol, and informed consent was obtained prior to specimen collection. For global metabolite profiling, ten samples from patients with each diagnostic category were used (10 AdenoCa, 10 SqCCa, 10 NM). For feature validation, specimens from an additional 49 patients were included. The demographics for all 79 patients are shown in Table 1.

Endobronchial and esophageal ultrasound-guided fine-needle aspiration (EBUS-TBNA and EUS-FNA) were used to biopsy lymph nodes of patients suspected to have lung cancer. A portion of each needle aspirate was plated on a slide for rapid on-site cytologic examination. The remainder was placed in saline, and all subsequent needle passes from the same site were placed in the same solution. For this study, 1 ml of fluid was pipetted from each sample prior to the addition of formalin for pathologic studies. The pipetted fluid was centrifuged at

200 g for 1 min to remove any cellular debris, and the supernatant was removed and frozen at -80°C . The presence or absence of malignant cells in the aspirate of each case and, if present, the type of malignancy were subsequently determined by a cytopathologist based upon morphology and immunohistochemistry of the pathology sample from which the fluid had been aspirated using WHO guidelines. The presence of representative malignant AdenoCa (A) and SqCCa (B) cells in aspirates is qualitatively shown in Fig. 1.

Sample preparation and data acquisition

Aspirate aliquots (50 μL) were thawed and diluted with 250 μL of ice-cold 50% methanol/0.2% formic acid (FA). Proteins were precipitated by adding 1050 μL ice-cold acetonitrile/0.2% FA, incubated at 4°C for 30 min and pelleted by centrifugation at 13,000g for 10 min at 4°C . Supernatants were transferred to clean 2 mL tubes and solvents were removed by speed vac.

Feature identification

Before sample analysis, the optimal injection volume was determined experimentally. A linear response of detectable features was observed with injections up to 8 μL , while injections of 10 μL and higher was accompanied with peaks that demonstrated saturation (data not shown). Therefore, 5 μL was chosen as the injection volume for all samples. Samples were analyzed in triplicate to obtain measures of technical reproducibility. Molecular features were extracted and aligned based on retention time and m/z , then deconvoluted.

For global liquid chromatography mass spectrometry (LC-MS) analysis, aliquots from a subset of samples (10 AdenoCa, 10 SqCCa and 10 NM) were reconstituted with 50 μL of ice-cold methanol/0.2% FA, then set on ice for 5 min before the addition of 50 μL H_2O /0.2% FA. A quality control (QC) sample was generated by pooling 5 μL from each of the 30 samples. Five μL from each sample were randomly injected in triplicate onto an LC-QqToF (Agilent, 1290 Infinity LC coupled to a 6538 QqToF). Additional QCs were injected before ($n = 8$), in-between ($n = 4$) and after ($n = 8$) the samples to monitor instrument performance. A Zorbax Eclipse C18 2.1×100 mm LC-column was operated with a linear gradient of 5% acetonitrile-0.01% FA to 95% acetonitrile-0.01% FA over 10 min. Data were acquired in positive ion mode over the mass-to-charge (m/z) range of 50 to 1600. Molecular features were extracted with MassHunter Molecular Feature Extractor. Extraction windows for retention time (RT) and m/z values were set based on peak width and m/z peak resolution. Features with intensity count greater than 5000, which represented an approximate signal-to-noise ratio > 20 , were imported into MassProfilerProfessional™, and peak alignment was performed based on log-2 values.

Pre-analytic filtering

Multivariate statistical analyses were performed. In brief, features with $m/z < 100$ and > 1000 were removed to limit analysis to small molecules. Additionally, features with RTs below 1 min and above 12 min were removed. Data for each injection were normalized to the sum of 11 features found in all injections (103.0997, 183.0662, 187.9556, 212.9992, 270.1919, 310.2509, 386.1091, 477.1407, 504.1505, 522.3632 and 624.207). Non-detects

were set to an intensity count of 4999 to minimize the difference between samples with a particular feature being absent versus present as described by Helsel [7]. Features reliably detected in all three replicate injections were averaged (Filter A). Features present in the 60%, 70%, and 80% cutoff analysis groups were retained for multivariate analyses (Filter B).

Multivariate analyses

Partial least squares discriminant analysis (PLS-DA) and orthogonal PLS-DA (OPLS-DA) analyses were run using the muma package in R. Data were normalized and scaled using the “Range” setting, as this method was found to be magnitude-independent with respect to both mean-values and fold-changes as established by van den Berg et al. [8]. At each level of Filter B, radii of both the combination of PLS-DA loading components and the combination of p1 matrix and pcorr1 matrix components were calculated. Percentile ranks of features based on radii within each analysis (PLS-DA and OPLS-DA) were calculated. For PLS-DA and OPLS-DA analyses, the mean ranks and standard deviations of rank across the three iterations (Filter B cutoff levels of 60%, 70% and 80%) were calculated and summed (mean + SD). Features with mean + SD of rank in the top half (lower ranks) of all PLS-DA and OPLS-DA iterations were selected. Utilization of various analysis variables (Filter A, B and PLS-DA and OPLS-DA) resulted in 12 individual analyses and multiple ranking scores for each feature. From these, consensus features were selected that consistently scored high in group separations. Finally, features were selected that had a > 1.1 fold difference in at least one of the pairwise comparisons between the three groups.

Feature validation

For feature validation, the m/z of two of the features was explored using the Human Metabolome Database. Features with m/z of 209.1 and 613.3 were tentatively identified as kynurenine and oxidized glutathione, respectively. These features were further investigated in a second set of lymph node aspirates. The metabolites tryptophan, glutamine, glutamate, and reduced glutathione were also quantitated because they are biochemically linked with kynurenine and oxidized glutathione. An LC-MS/MS method was set up for specific quantitation of reduced (GSH), oxidized glutathione (GSSG), kynurenine, tryptophan, glutamine (Gln) and glutamate (Glu) to determine whether relative concentrations of these metabolites were associated with lymph node aspirates positive for malignancy. Five μ L from each sample were randomly injected onto an LC-QQQ (Agilent, 1290 Infinity LC coupled to a 6490 Triple Quad). A Poroshell 120 EC-C18 2.1 $\times$ 150 mm column was operated with a linear gradient of 2% methanol/0.01% FA to 80% methanol-0.01% FA over 10 min, and then returned to starting conditions. Data were acquired in multiple reaction monitoring mode, monitoring the ion transitions of 308 to 162, 613 to 355, 209 to 94, 205 to 146, 147 to 84, and 148 to 84 for GSH, GSSG, kynurenine, tryptophan, Gln and Glu, respectively. Metabolite validation and peak areas were determined using Agilent QQQ Quantitative Analysis software. Metabolites were quantified using peak area, at the right retention time against an external standard curve.

Results

Feature identification

A total of 142,789 features were recognized, 35,039 of which were observed at least twice. Features with m/z ratio of > 100 and < 1000 and a retention time between 1–12 min were retained, leaving 10,116 features for analysis. To be considered clinically relevant, features were required to be present in all three technical replicates (filter A) and in the majority of specimens from at least one clinical group (AdenoCa, SqCCa, NM; filter B). Application of 60%, 70%, and 80% cutoff criteria revealed 188, 150, and 111 features, respectively reliably detected in at least one patient group (see Methods). The selected features were analyzed by PLS and OPLS. Both multivariate statistical analyses showed separation of the patient groups (Figs. 2A and B), although there were two AdenoCa patients that grouped with the NM group.

Highest ranking consensus features, with a minimum of 1.1 fold-difference in at least one of the pair-wise comparisons between AdenoCa and SqCCa or between malignant and NM, are listed in Table 2. Five features separated malignant from NM categories with a $p < 0.05$. Eleven features separated AdenoCa from SqCCa with a $p < 0.05$.

Feature validation

The kynurenine-tryptophan ratio (KTR) was 2.8-fold higher in malignant versus NM samples ($p = 0.0017$, Student's t-test) (Table 3). Reduced and oxidized glutathione levels were significantly higher in malignant compared to NM aspirates (4.62 and 2.53-fold, respectively; $p < 0.05$, Student's t-test). The selected metabolites analyzed by targeted tandem mass spectrometry produced qualitatively similar results to those obtained in the global metabolite analysis. In addition, we observed statistically significant differences in the concentrations of Gln and Glu and in the Gln/Glu ratio in positive versus negative aspirates.

Discussion

In this study, we demonstrate that lymph node aspirates from lung cancer patients contain diagnostically relevant metabolites. Two panels of consensus features were identified that distinguish between AdenoCa, SqCCa, and NM with $> 93\%$ accuracy (Table 2). Two of these features were identified, validated, and shown to be involved in biochemical pathways that differ for cancer patients compared to NM patients. The data from this proof-of-principle study confirm the viability and relevance of metabolite analysis of lymph node aspirates.

Lymph node aspirates are a novel source for metabolomic diagnosis. The material for analysis was obtained during routine diagnosis and staging of lung cancer with a relatively non-invasive tool (endoscopy), and the data were generated without diversion of specimen from standard clinical analysis. It was not evident *a priori* that the lymph node aspirates would be a source of reproducible and clinically relevant data because numbers of malignant (and therefore their metabolites) and non-malignant cells (lymphocytes, bronchial epithelial cells, red blood cells) were unquantifiable. The multivariate data analyses, however, that mined multi-dimensional relationships between metabolite features, produced a list of

features (Table 2) that consistently distinguished patient groups despite these variables (Fig. 2).

The identification of metabolite patterns characteristic to lung cancer offers insight into the biochemical pathways unique to or disproportionately altered in lung cancer, and thereby offers insight into potential therapeutic targets. Our validation studies confirm that the feature with m/z of 209.2 was indeed kynurenine. While kynurenine alone was not significantly different between MAL and NM, its precursor tryptophan was found to be significantly lower in MAL aspirates, suggesting increased utilization of tryptophan by the metastatic cells. Using the kynurenine/tryptophan ratio (KTR) to normalize for sample variability resulted in an even greater fold-change difference with a better p-value. This is in agreement with previous reports of kynurenine and tryptophan in serum from lung cancer patients [9–11]. These and earlier studies suggest that tumor cells actively take up tryptophan, convert it to kynurenine, and excrete kynurenine to inhibit T-cell-mediated immune response, thereby promoting tumor growth and aggressiveness.

Similarly, the feature with m/z of 613.3 was correctly identified as GSSG. Glutathione concentrations are known to be 2-fold higher in lung tumor tissue than in adjacent normal tissue [12,13]. Therefore, it was not surprising that oxidized glutathione came up as one of the consensus features. In the validation studies, reduced and oxidized glutathione levels were shown to be higher (4.6-fold and 2.5-fold, respectively) in lymph node aspirates harboring malignant tumor cells than in NM aspirates (Table 3, Fig. 3). This clearly confirms the importance of glutathione synthesis in lung cancer metabolism. The relative amount of glutamine was reduced by 30% in aspirates harboring malignant tumor cells compared to negative aspirates, while glutamate increased by 40%, suggesting active glutaminolysis by malignant tumor cells. This is in agreement with previous reports showing glutamine dependence of lung tumors [12,14,15].

There were some limitations to this study. The metabolomic platform chosen is biased toward compounds that ionize well in positive ionization mode. Very hydrophilic compounds or compounds unsuitable to positive electro-spray ionization might have been excluded methodologically. While individual feature groups might have been lost, the results demonstrate that clinically meaningful data were retained.

Conclusion

In conclusion metabolomic analyses of lymph node aspirates obtained during routine clinical practice identified diagnosis-specific metabolites. Validation of selected consensus features further suggests that pathways specific to malignancy may be identified, a finding with potential therapeutic implications. This proof-of-concept study provides data to support further metabolomic research using lymph node aspirates and supports the creation of a lymph node aspirate metabolite database.

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Clinical practice

- The 5-year survival rate of patients with lung cancer, compared with other cancers, has been poor and essentially has not changed over the last 20 years.
- The poor survival of lung cancer patients has been attributed to the high rate of metastatic disease and major advances in patient survival can be made by actively targeting metastasis.
- It is hypothesized that the metabolic activity of lung tumor cells found in lymph node aspirates will correlate with tumor type, stage, and patient survival.
- Knowledge of the biochemical and physiological adaptations of metastatic tumor cells is expected to be applicable to improving and refining therapy selection to effectively treat lethal metastasis and improve patient survival.

Fig. 1. Representative Diff-Quick stains of aspirate smears. (A) Shows neoplastic cells with enlarged nuclei, irregular nuclear membranes, and multiple small nucleoli. The arrangement of the cells and the cytologic features are representative of adenocarcinoma. (B) Shows sheets of neoplastic squamous cells with focal areas of keratinization. Both images are 400 × magnification.

Fig. 2. Representative OPLS-DA (A) and PLS-DA (B) scatter plots using a 60% cutoff group. Red circles = SqCCa, Black triangles = AdenoCa, Green squares = NM.

Fig. 3. Scatter plot of (A) KYN(μ M)/TRY(nM), (B) GSSG/GSH and (C) Gln/Glu ratios in lymph node aspirates.

Table 1

Patient demographics.

	Adenocarcinoma (AdenoCa)	Squamous cell carcinoma (SqCCa)	Non-malignant (NM)	Total
Diagnosis	31 (40%)	19 (24%)	29 (36%)	79 (100%)
Stage I	-	-	-	-
II	7 (22%)	1 (5%)	-	8 (10%)
III	15 (48%)	9 (47%)	-	24 (30%)
IV	9 (29%)	9 (47%)	-	18 (23%)
Gender				
Male	17 (55%)	14 (78%)	15 (52%)	46 (58%)
Female	14 (45%)	5 (22%)	14 (48%)	33 (42%)
Smoking status				
Yes	31 (100%)	18 (95%)	22 (76%)	71 (90%)
No	0 (0%)	1 (5%)	7 (24%)	8 (10%)
Age	62.0 ± 10.7	66.0 ± 12.6	62.2 ± 14.2	63.0 ± 12.6
Ethnicity				
Caucasian	26	19	26	71 (90%)
African American	5	0	0	5 (6%)
Hispanic/Latino	8 (10%)			8 (10%)

Table 2

Shown are two sets of 24 representative consensus features separating MAL from NM samples or AdenoCa from SqCCa samples.

Feature [m/z]	MAL vs. NM		AdenoCa vs SqCCa	
	Fold Change ^a	p-value	Feature [m/z]	Fold Change ^a p-value
200.12	-3.92	0.05*	966.55	-7.78 0.05*
158.08	-3.41	0.04*	554.16	-4.03 0.13
966.55	-3.39	0.09	128.09	-3.16 0.22
212.15	-3.34	0.04*	200.12	-3.16 0.43
299.18	-3.32	0.09	838.44	-2.85 0.5
712.71	-3.23	0.09	712.71	-2.66 0.5
838.44	-2.97	0.23	158.08	-2.64 0.52
554.16	-2.81	0.15	212.15	-2.45 0.62
128.09	-2.57	0.28	299.18	-2.33 0.72
392.20	-2.17	0.42	452.34	2.11 0.48
612.33	-2.13	0.83	209.14	2.28 0.03*
308.21	1.99	0.94	434.33	2.28 0.03*
322.15	2.08	0.69	208.16	2.28 0.03*
167.13	2.10	0.68	181.15	2.30 0.03*
434.33	2.13	0.28	325.27	2.30 0.02*
208.16	2.13	0.29	308.21	2.39 0.38
325.27	2.14	0.23	239.22	2.41 0.0012***
181.15	2.14	0.21	286.08	2.57 0.01*
209.14	2.19	0.15	322.15	2.64 0.04*
239.22	2.23	0.07	490.31	2.69 0.08
286.08	2.30	0.19	392.20	2.99 0.05
452.34	2.33	0.009**	348.18	3.07 0.01*
490.31	2.55	0.18	167.13	3.41 0.010**
348.18	4.92	0.0031***	612.33	3.63 0.01*

^aFold Change relative to NM and AdenoCa, respectively.

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Table 3

Relative fold changes (FC) of selected metabolites between MAL and NM and between AdenoCa and SqCCa.

	MAL/NM		AdenoCa/SqCCa	
	FC	p-value	FC	p-value
KYN	1.36	0.139	0.97	0.92
TRP	0.59	0.0027**	0.98	0.92
KYN/TRY	2.80	0.0017***	1.01	0.98
GSH	4.62	0.043*	1.54	0.57
GSSG	2.53	0.0009***	1.57	0.17
GSSG/GSH	1.60	0.107	2.27	0.053
Gln	0.69	0.0076**	0.92	0.70
Glu	1.44	0.045*	1.10	0.74
Gln/Glu	0.54	0.0001***	0.71	0.13

KYN kynurenine.

TRP, tryptophan.

GSH, reduced glutathione.

GSSG, oxidized glutathione.

Gln, glutamine.

Glu, glutamate.

*
p < 0.05.

**
p < 0.001.

p < 0.002.