

ORIGINAL ARTICLE

Differential diagnosis of lung cancer and benign lung lesion using salivary metabolites: A preliminary study

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Funding information

YU-COE(C) from Yamagata University; the Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI, Grant/Award Numbers: 16K11742, 17K11897, 19K10304

Abstract

Background: Saliva is often used as a biomarker for the diagnosis of some oral and systematic diseases, owing to the non-invasive attribute of the fluid. In this study, we aimed to identify salivary biomarkers for distinguishing lung cancer (LC) from benign lung lesion (BLL).

Materials and Methods: Unstimulated saliva samples were collected from 41 patients with LC and 21 with BLL. Salivary metabolites were comprehensively analyzed using capillary electrophoresis mass spectrometry. To differentiate between patients with LCs and BLLs, the discriminatory ability of each biomarker was assessed. Furthermore, a multiple logistic regression (MLR) model was developed for evaluating discriminatory ability of each salivary metabolite.

Results: The profiles of 10 salivary metabolites were remarkably different between the LC and BLL samples. Among them, the concentration of salivary tryptophan was significantly lower in the samples from patients with LC than in those from patients with BLL, and the area under the curve (AUC) for discriminating patients with LC from those with BLL was 0.663 (95% confidence interval [CI] = 0.516–0.810, $p = 0.036$). Furthermore, from the MLR model developed using these metabolites, diethanolamine, cytosine, lysine, and tyrosine, were selected using the back-selection regression method. The MLR model based on these four metabolites had a high discriminatory ability for patients with LC and those with BLL (AUC = 0.729, 95% CI = 0.598–0.861, $p = 0.003$).

Conclusion: The four salivary metabolites can serve as potential non-invasive biomarkers for distinguishing LC from BLL.

KEYWORDS

benign lung lesion, lung cancer, metabolites, saliva

INTRODUCTION

Lung cancer (LC) is one of the most prevalent cancers globally and the leading cause of cancer-related deaths among men.¹ LC can be classified into non-small cell LC (NSCLC), which accounts for ~80% of the cases, and small-cell lung cancer, which accounts for ~20% of the cases. NSCLC is often asymptomatic or presents with only nonspecific symptoms during its early stages. The five-year survival rate for

NSCLC is only 15%, partly because the disease is usually diagnosed at a late stage, when it is frequently metastatic and incurable.^{2,3} Early detection is crucial for reducing the morbidity and mortality associated with NSCLC. However, it is difficult to promote the current approaches for detecting NSCLC (such as computed tomography [CT], positron emission tomography [PET], or magnetic resonance imaging) owing to their high costs and radiation exposure, as well as low positive-detection rates during the early stages of the

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disease. Therefore, new practical and effective approaches for the early diagnosis of NSCLC are desperately needed.

Tissue specimens and blood samples have been widely used for the diagnosis of NSCLC and for research.⁴ However, human saliva is increasingly being used as a diagnostic specimen for various diseases, because its collection is convenient and non-invasive. As analytical technologies become more advanced, salivary biomarkers derived from genomics, transcriptomics, proteomics, microbiomics, and metabolomics have been reported as biomarkers for the clinical screening and detection of LC.^{5–10} However, most of these studies analyzed differences between LC and controls, and there are not many reports that discriminate LC from benign lung lesions (BLL).^{11,12} To the best of our knowledge, there has only been one study based on salivary metabolomic approaches for the discrimination of LC from BLL.¹³

However, the identified metabolites were analyzed using a semi-automatic biochemical analyzer and the analyzed number of compounds was limited and focal.¹³ There are currently no reports on salivary metabolite biomarkers for discriminating LC from BLL using the comprehensive metabolomic approaches based on the mass spectrometry-based metabolomics. In the present study, we aimed to identify salivary metabolite biomarkers that can distinguish benign tumors from malignant tumors of the lung, using comprehensive mass spectrometry-based metabolomics.

METHODS

All procedures performed in the current study involving human participants were conducted according to the ethical standards of the institutional and/or national research committee and the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. The Institutional Review Board (IRB) Ethics Committee of Yamagata University, Faculty of Medicine, Yamagata, Japan, approved the protocol used in the study (IRB H27-288, H28-18, H29-180, 2018-455, 2019-251). Written informed consent was obtained from all participants before specimen collection. For the perioperative oral management, the patients with pulmonary lesions had consulted the Department of Dentistry, Oral, and Maxillofacial Plastic and Reconstructive Surgery, Yamagata University Hospital from 2016 to 2017. This study included patients who consulted a dental surgeon before lung surgery or underwent PET/CT for LC. According to the study design, whole unstimulated saliva samples were collected from patients with pulmonary lesions, but not from healthy individuals. Participants were selected and enrolled upon confirmation of clinical or pathological diagnosis. All enrolled participants at the time of pathological or clinical diagnosis of benign pulmonary nodules or LC reported having no history of malignancy and no prior treatment, such as chemotherapy or radiotherapy. The LC group included patients with a pathological diagnosis of LC, whereas the BLL group included patients who were diagnosed with granuloma or inflammatory changes on

pathology or shrinkage during follow-up. Consequently, 42 patients with LC and 21 patients with BLL from the respective groups were selected for the concluding statistical analysis.

Saliva collection and sample preparation

All participants were asked to refrain from eating and drinking for at least 1.5 hours before saliva collection. Participants rinsed their mouths with water and were instructed to spit saliva into 50-cc Falcon tubes kept in paper cups filled with crushed ice. Approximately 4–5 mL unstimulated whole saliva was collected from each participant over a 5- to 15-minute interval. After collection, the saliva samples were processed according to a standard operating procedure previously described, which included centrifugation and the addition of reagents to the supernatants.¹⁴ The processed saliva samples were immediately stored at -80°C .

Metabolomic analysis of saliva

The processing of individual saliva samples for metabolomic analysis was performed as previously described.^{15,16} Briefly, frozen saliva samples were thawed at room temperature and then centrifuged using a 5-kDa cutoff filter (Millipore) at $9100 \times g$ for at least 2.5 hours at 4°C to remove macromolecules. A 45- μL aliquot of filtrate from each sample was removed and added to 1.5 mL Eppendorf tubes to which 5 μL of water containing 2 mM methionine sulfone, 2-(N-morpholino) ethane sulfonic acid (MES), D-camphor-10-sulfonic acid, sodium salt, 3-aminopyrrolidine, and trimesate was added and mixed. Capillary electrophoresis time-of-flight mass spectrometry

TABLE 1 Characteristics

	<u>Benign tumor</u> <i>n</i> = 21	<u>Lung cancer</u> <i>n</i> = 42
Age, y		
Min–max (median)	43–86 (62)	39–86 (63)
Sex		
Male	15	28
Female	6	14
Pathological findings		
Adenocarcinoma		33
Squamous cell carcinoma		7
Neuroendocrine tumors		1
Small cell carcinoma		1
Stage		
I		31
II		4
III		4
IV		3

TABLE 2 Differences in the salivary metabolites in samples from patients with LC and those with BLL^a

Metabolites	LC (n = 42)		BLL (n = 21)		p-value	AUC	p-value
	Mean	SD	Mean	SD			
Choline	7.222	5.601	8.703	4.58	0.091	0.632	0.091
Diethanolamine	0.382	0.503	0.162	0.321	0.094	0.612	0.149
Cytosine	0.073	0.178	0.155	0.238	0.128	0.586	0.268
Thymine	0.363	0.702	0.902	1.582	0.116	0.601	0.194
Isoleucine	5.068	6.119	6.542	5.381	0.122	0.620	0.122
Leucine	9.757	10.445	12.491	9.768	0.119	0.621	0.119
Lysine	60.425	42.39	93.098	82.711	0.122	0.620	0.122
Phenylalanine	16.016	13.924	19.338	10.409	0.085	0.634	0.085
Tyrosine	26.37	24.598	28.273	16.824	0.129	0.618	0.129
Tryptophan	1.488	1.242	2.093	1.182	0.036	0.663	0.036

Note: The AUC values of the discriminatory ability of the metabolites are also presented ($\mu\text{mol/L}$).

Abbreviations: AUC, area under the curve; BLL, benign lung lesion; LC, lung cancer; R5P, ribose 5-phosphate.

^aRepresentative salivary metabolites with a *p*-value of <0.15 in Mann–Whitney *U*-test.

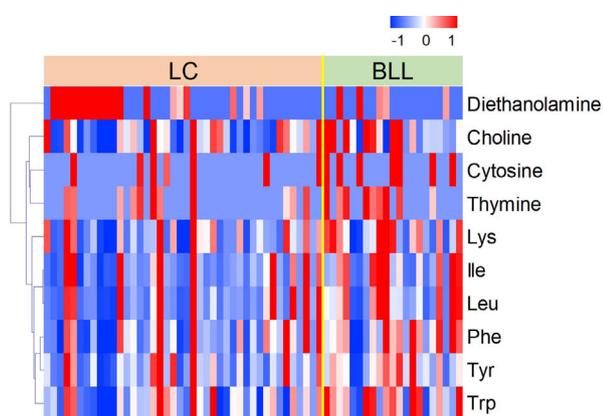


FIGURE 1 Heatmap of 10 salivary metabolomic profiles. The heatmap depicts the differences between the LC and BLL groups ($p < 0.15$, Mann–Whitney *U*-test). Colors represent concentration of individual metabolites divided by the *z*-score of the LC and BLL salivary samples. Red and blue represent higher and lower concentrations, respectively. The vertical axis indicates the number of patients. The LC group is indicated to the left of the yellow line and the BLL group to the right of the yellow line. The horizontal axis indicates the salivary metabolites. BLL, benign lung lesion; LC, lung cancer

(CE-TOFMS) was used to quantify the charged metabolites in the positive and negative modes.^{15,16} CE-TOFMS raw data were processed using our proprietary software MasterHands.¹⁷ Metabolite concentrations were calculated as previously described based on peak areas normalized to the internal standards that were added to the samples and on peak areas of a mixture of standard compounds normalized to the mixed internal standards.^{15,16}

Statistical analyses

To determine the ability of salivary metabolites to distinguish between patients with benign lung tumors and LC, a

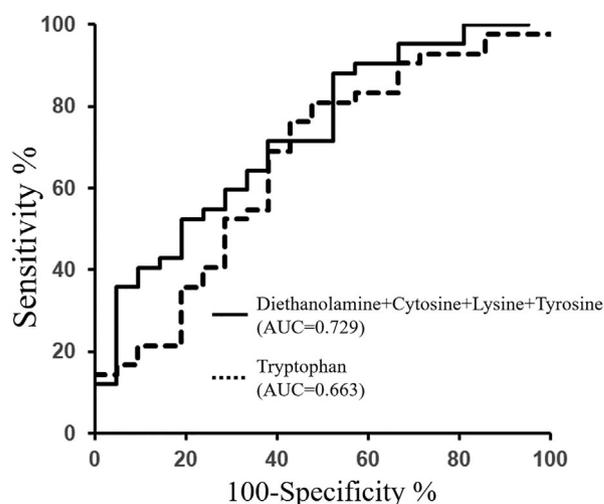


FIGURE 2 Receiver operating characteristic (ROC) curves of tryptophan (Trp) and the multiple logistic regression (MLR) models based on the salivary concentration of diethanolamine, cytosine, lysine, and tyrosine for their discriminatory ability between patients with LC from those with BLL. The area under the curve (AUC) is 0.663 (95% CI = 0.516–0.810; $p = 0.036$) and 0.729 (95% CI = 0.598–0.861; $p = 0.003$) respectively. BLL, benign lung lesion; LC, lung cancer

multiple logistic regression (MLR) model was developed. First, metabolites detected in more than 30% of the saliva samples from at least one group of participants (BLL or LC) were selected. Second, Mann–Whitney *U*-test was conducted to identify the candidate salivary metabolites that were different between the two groups. Finally, to determine the ability of salivary metabolites to distinguish between patients with LC and those with BLL, a MLR model was developed using the candidate metabolites obtained from the Mann–Whitney *U*-test. MLR analysis of the above candidate metabolites was performed using a backward elimination regression method for multivariate logistic analysis. The

TABLE 3 ROC analysis of four salivary metabolites using the MLR models

Metabolites	AUC (LC vs. BLL)	<i>p</i> -value	95% CI		
			Low	High	
Diethanolamine + cytosine + lysing + tyrosine	0.729	0.003	0.598	–	0.861

Abbreviations: BLL, benign lung lesion; LC, lung cancer; MLR, multiple logistic regression; ROC, receiver operating characteristic.

predictive performance of each metabolite and the MLR models were evaluated using the area under the curve (AUC) of the receiver operating characteristic (ROC) curve. Statistical analyses were performed using SPSS version 20 software (SPSS). Heatmaps were generated using XLSTAT software (Data Analysis and Statistical Solutions for Microsoft Excel, Addinsoft).

RESULTS

Data regarding the age, sex, and pathological findings of the participants are shown in Table 1. Table 2 shows the representative metabolites with $p < 0.15$ as determined by the Mann–Whitney *U*-test. The concentration of tryptophan (Trp) was significantly lower in the saliva samples of the LC group than in those of the BLL group ($p = 0.036$). Although the concentrations of eight metabolites, namely choline, thymine, cytosine, phenylalanine (Phe), leucine (Leu), isoleucine (Ile), lysine (Lys), and tyrosine (Tyr), were higher in the saliva samples of the LC group than in those of the BLL group, the difference was not significant. The concentration of diethanolamine was lower in the saliva sample of the LC group than in that of the BLL group; however, the difference was not significant. Table 2 also shows the AUC value of the metabolites for distinguishing between BLL and LC. From all the assessed candidate metabolites, the AUC value of Trp was highest for discriminating BLL from LC (AUC = 0.663, 95% CI = 0.516–0.810, $p = 0.036$). Figure 1 shows the heatmaps of the 10 abovementioned salivary metabolites. Most of the metabolites had a lower *z*-score in the LC group compared with those in the BLL group. Figure 2 shows the ROC curves of Trp and the MLR models, and Table 3 shows the AUC values from the MLR models. The MLR model was developed based on the four metabolites diethanolamine, cytosine, Lys, and Tyr, which were selected using the back-selection regression method. These metabolites were used to evaluate the potential of the multivariate logistic analysis to distinguish between BLL and LC. The ability of the model to discriminate BLL from LC using a combination of the four metabolites was 0.729 (95% CI = 0.598–0.861; $p < 0.003$).

DISCUSSION

Our current findings demonstrated that salivary metabolites could help distinguish LC from BLL. To the best of our knowledge, this study is the first to compare patients with

LC with those having BLL. Clinically, it is important to distinguish LC from BLL. General physicians must decide whether to perform a bronchial biopsy on a patient with lung lesions or whether the patient should be referred to a surgeon. Given the importance of this decision, our results are significant.

To the best of our knowledge, the present investigation is the first comprehensive mass spectrometry-based metabolomic study for the discrimination of LC from BLL. However, differences in some metabolites between LC, BLL, and controls have been previously reported by Bel'skaya et al.¹³ Although the biochemical composition, including some metabolites, was not compared between BLL and LC, the biochemical composition, including some metabolites, was compared among three groups (controls, BLL, and LC). Imidazole compounds and sialic acids were significantly different among the three groups. Nevertheless, these were not validated for their potential to discriminate LC from BLL and their use as candidate metabolic biomarkers. In addition, despite their potential, these metabolites were not candidate biomarkers in the present study.

The discrimination ability of salivary metabolites, the AUC, between LC and BLL was 0.729 in this present study. In clinical practice, CT, PET, and PET/CT have been used to discriminate LC from BLL. Overall sensitivities of CT, PET, and PET/CT for discriminating LC from BLL are 82%, 88%, and 88%, respectively, whereas their specificities are 66%, 71%, and 77%, respectively. In diagnosing BLL and LC, PET/CT is significantly better with respect to specificity than PET or CT alone.^{18–20} In the present study, salivary screening of LC from BLL was not superior to diagnostic imaging with CT, PET, and PET/CT routinely used in the diagnosis of lung cancer. However, these imaging tests are expensive and associated with a risk of radiation exposure. Therefore, the ordering of PET/CT scans requires careful consideration by the physician and are typically only performed when symptoms are present. The most notable point of salivary biomarkers is their noninvasive feature. Therefore, salivary screening as a first step for discrimination of LC from BLL, leading to CT, PET, or PET/CT, may be effective. This approach may also be of use to general practitioners and might allow for the identification of more people with LC through non-invasive screening. Tests that are not affected by exposure and do not require invasive procedures are strong candidates as screening tests, but require further investigation.

In this present study, the salivary concentration of Trp was significantly lower in the samples from the LC group than in those from the BLL group. Although the mechanism

underlying this difference remains unclear, a previous study reported that serum Trp was significantly lower in patients with LC than in healthy controls.²¹ Trp, a precursor of kynurenine, is metabolized to kynurenine by the action of indoleamine 2,3-dioxygenase (IDO).^{22,23} Under normal physiological conditions, IDO expression is modulated; however, it is often upregulated in several cancer types. The upregulation of IDO expression leads to increased Trp metabolism that increases kynurenine production.^{24–27} Kynurenine, an oncometabolite, suppresses T-cell differentiation, and consequently, promotes cancer growth and development.^{22,23,28–31} This eventually leads to decreased serum Trp levels in patients with cancer. Considering the positive correlation between serum and saliva, our findings of significantly lower salivary Trp concentration in the samples from patients with LC than in those from patients with BLL are reasonable and supported, at least in part, by the abovementioned study.²¹ The mechanisms elaborated above possibly also underlie the results of the present study. However, the exact explanation for the difference in salivary Trp concentrations could not be deduced; this warrants further studies and validation.

This study has several limitations. First, we collected saliva from participants with BLL and LC, but not from healthy controls. Although our data suggested that salivary metabolites were significantly lower in patients with BLL than in those with LC, future studies should also examine the levels of salivary metabolites in healthy control subjects. Second, the numbers of participants in the BLL and LC groups in this study were small. Well-powered studies are needed in the future to precisely identify the salivary metabolites that can be used to accurately distinguish LC from BLL. The third limitation is the lack of external validation. This shortcoming is related to the second limitation, because a larger sample size is necessary for external validation in the future. Finally, comorbidities were not accounted for in this study. Because several oral or systemic conditions may be associated with changes in saliva markers, it would be important to account for such comorbidities in future studies.

CONCLUSIONS

This was the first study to identify salivary metabolites for distinguishing LC from BLL. The salivary metabolite profiles between patients with BLL and LC were found to be significantly different. For physicians who are not lung surgery specialists, the ability to distinguish LC from BLL is very important. The use of salivary metabolites as diagnostic biomarkers may emerge as an important non-invasive means of distinguishing LC from BLL.

ACKNOWLEDGMENTS

We thank all the patients who provided the samples, as well as Editage for editing our article. This work was supported by grants from YU-COE(C) from Yamagata University and

the Ministry of Education, Culture, Sports, Science and Technology (MEXT) KAKENHI (16K11742, 17K11897, and 19K10304).

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

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How to cite this article: Takamori S, Ishikawa S, Suzuki J, Oizumi H, Uchida T, Ueda S, et al. Differential diagnosis of lung cancer and benign lung lesion using salivary metabolites: A preliminary study. *Thorac Cancer*. 2022;13:460–5. <https://doi.org/10.1111/1759-7714.14282>