A metabolomics strategy to identify potential biomarkers associated with human laryngeal cancer based on dried blood spot mass spectrometry approach

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Abstract
Laryngeal cancer (LC) as one of common malignant tumors in the head and neck region accounted for 1% to 5% of new cancer cases and was ranked as the third otolaryngology cancer. However, some patients with LC were diagnosed at the advanced stage, which can cause delayed diagnosis and treatment. It is an urgent task to seek effective biomarkers for the early diagnosis of LC aimed at alleviating suffering. A combination of dried blood spot sampling and direct infusion mass spectrometry technology was applied to 39 patients with LC and 53 healthy individuals. Multiple algorithms towards 93 metabolites including amino acids and carnitine/acylcarnitines were run for selecting differential metabolites. Furthermore, leave-one-out cross-validation method was used to evaluate diagnostic performance of selected metabolite biomarkers. A biomarker panel consisting of arginine, proline, hexacosanoic carnitine, ornithine/citrulline, and 3-hydroxy-octadecenoylcarnitine exhibited potential to distinguish patients with LC from healthy individuals, with a sensitivity of 0.8974 and a specificity of 0.8302 in leave-one-out cross-validation model.

The metabolic analysis of LC patients is beneficial to screen disease-associated biomarkers and develop new diagnostic approaches.

Abbreviations: Arg = arginine, C18:1-OH = 3-hydroxy-octadecenoylcarnitine, C26 = hexacosanoic carnitine, Cit = citrulline, CPT = carnitine palmitoyl transferase, DBS = dried blood spot, FC = fold change, HC = healthy control, LC = laryngeal cancer, LOO = leave-one-out, MS = mass spectrometry, Om = ornithine, PLS-DA = partial least squared discriminant analysis, Pro = proline, SAM = significant analysis of microarrays, VIP = variable importance in projection.

Keywords: biomarker, dried blood spot, laryngeal cancer, mass spectrometry, metabolomics

1. Introduction
Laryngeal cancer (LC) as the largest subgroup of head and neck cancers was ranked the 21st in overall cancers.\textsuperscript{[1]} LC with crude incidence rate of 1.86/100,000 explains approximately 85% to 90% of malignant tumors of the larynx.\textsuperscript{[2]} Approximately 13,150 diagnosed new cases of LC occurred every year in the USA.\textsuperscript{[3]} LC patients were usually diagnosed at the advanced stage, mostly for the nonspecific symptoms caused by LC, which can lead to delayed diagnosis and treatment.\textsuperscript{[4]} Surgical treatment, chemotherapy and radiotherapy could be used in...
the treatment of LC, whereas these therapeutic methods may result in adverse reactions including the need of definitive tracheostomy and voice loss, which seriously influence the quality of life for the patients with LC.\[5\] Hence, it is an urgent task to seek an effective tool for the early diagnosis of LC to alleviate suffering.

The usage of biomarkers is a possible way to improve the early diagnosis of LC. Metabolomics is based on the quantitative measurement of low-molecular-weight metabolites in organisms, and can assess a broad range of metabolites simultaneously. Metabolomics has been dedicated to biomarker discovery and interpretation of biochemical pathways for many diseases, to improve disease diagnosis, therapy and prognosis.\[6,7\] Abnormal metabolism has been considered as a universal characteristic of cancer cells.\[8\] Currently, abnormal metabolites in cancer patients has been inspected via Metabolomics approach.\[9,10\] Mass spectrometry (MS) is one of the most commonly used techniques in metabolomics.\[11,12\] It can provide accurate mass data for a large number of metabolites simultaneously, especially for multiple low-abundance metabolites.\[13,14\] This technology has been applied to study metabolites in serum of patients with LC, and some metabolites showed significant differences between LC and healthy control (HC) groups.\[15\] Dried blood spot (DBS) sampling technology is usually used in newborn screening, and this technology is beneficial to the development of cancer diagnosis.\[16\] Comparing to traditional venous blood sampling, this technology can make specimen easy to transport and store.\[17\] The combination of MS and DBS sampling technology has been applied to characterize the changes of metabolic profiles for several kinds of diseases, and can provide high-throughput and sensitivity analysis of metabolites.\[17\] In this study, metabolomic profiles for DBS samples of patients with LC and healthy individuals were explored by MS strategy to seek effective biomarkers for the diagnosis of LC. Multiple statistical methods were used to screen biomarkers for LC. Finally, 5 biomarkers were selected to distinguish patents with LC from healthy individuals, which were used to build a logistic regression prediction model with high sensitivity and specificity.

2. Materials and methods

2.1. Study design and participants

In the present study, a total of 92 participants, including 53 HC individuals and 39 patients with LC, were enrolled from the First Affiliated Hospital of Jinzhou Medical University. There were no age \((P = .4702, \text{Student } t \text{ test})\) and gender differences \((P = .7182, \text{Fisher exact test})\) between patients with LC and healthy individuals. All recruited patients were diagnosed with LC, and no one suffered from other types of malignant diseases, acute diseases, and other diseases which could influence biological indicators. The diagnosis and other information for patients with LC were acquired from inpatient database in the First Affiliated Hospital of Jinzhou Medical University. Patients with missing data were excluded from the analysis. This study was approved by the Ethics Committee of the First Affiliated Hospital of Jinzhou Medical University. Written informed consent was obtained from participants.

2.2. Blood sample collection and pretreatment

To eliminate the disturbance of diet, blood samples were collected after an overnight fasting. DBS specimen was obtained by fingertip puncture. A disc with 3 mm diameter was punched from DBS paper. The disc was put into Millipore MultiScreen HV 96-well plate (Millipore, Billerica, MA) for extracting metabolites. All standards including amino acids and acylcarnitines were dissolved in 2mL pure methanol, and stored at 4°C. A 100-fold dilution of working solution was prepared for metabolite extraction. 100μL of working solution was added into each well containing a DBS disc, and shaking for 20 minutes. The sample was centrifuged at 1500 x g for 2 minutes. Then, new flat-bottom 96-well plates were used to collect filtrate. Two low-level and 2 high-level quality control solutions were randomly inserted in each plate to check the stability of large-scale analysis. Quality control and filtrate solutions were dried in pure nitrogen gas flow at 50°C. Afterward, these samples were derivatized with 60 uL mixture of acetyl-chloride/1-butanol (10:90, v/v) at 65°C for 20 minutes. Subsequently, these derivatized samples were dried again. The dried samples were mixed with 100 uL fresh mobile phase solution for metabolomic analysis.

2.3. Metabolomic analysis

The direct injection MS metabolomic analysis was performed using an AB Sciex 4000 QTrap system (AB Sciex, Framingham, MA). All the analytes were scanned under positive electrospray ionization mode, and detailed scan parameters were referred to previous report.\[18\] The sample injection volume was 20 uL. 80% ACN aqueous mixture was used as mobile phase. In gradient elution program, flow rate was set as 0.2 mL/min at initial stage, then decreased to 0.01 mL/min within 0.08 minutes, maintained constant until 1.5 minutes, subsequently returned to 0.2 mL/min within 0.01 minute, and maintained constant for another 0.5 minute. The ion spray voltage was set at 4.5 kV. Auxiliary gas temperature was 350°C.

System control and data collection were performed using analyst v1.6.0 software. ChemoView 2.0.2 (AB Sciex) was applied for data preprocessing. Ninety-three parameters composed of 23 amino acids, 26 carnitine/acylcarnitines, 44 derivatives, and related ratios\[18\] were obtained for following analysis. An unsupervised model of principal component analysis was used to evaluate holistic metabolic alterations between LC and HC groups. An supervised model of partial least-squared discriminant analysis (PLS-DA) was applied to distinguish laryngeal patients from healthy individuals and to obtain valuable importance in projection (VIP) values to evaluate important contributions of metabolites to classification by SIMCA-P v12.0 (Umetrics, Umea, Sweden). A permutation test with 200 times was used to assess the risk of over-fitting for this model. The Student \(t\) test and Mann–Whitney \(U\) test were carried out to conduct differential assessment toward parametric and nonparametric variables, respectively. False discovery rate based on Benjamini Hochberg procedure was estimated. Volcano plots were created aimed to select important metabolites with VIP \(>1\), fold change (FC) \(>1.2\) or \(<-1.2\), and adjusted \(P\) value (adj \(P\) value) \(<.05\) in LC patients compared with healthy individuals. Significant analysis of microarrays (SAM) was applied to further screen significantly changed metabolites between LC and HC groups by R v3.3.1. For selecting independent variables, stepwise logistic regression analysis was conducted. Ultimately, binary logistic regression model was established based on selected independent variables for differentiating LC from HC groups. Leave-one-out (LOO) cross-validation method was adopted to testify the predictive ability of this model. Specificity and
sensitivity were calculated for each prediction model in this study. The statistical analysis was performed using SAS statistical software.

3. Results

3.1. Demographics of study cohort

In this study, a total of 92 blood samples were collected to define biomarker candidates for LC. Fifty-three healthy individuals (mean age 61.15 ± 8.47, range 47–85 years) were recruited including 49 (92.5%) males and 4 (7.5%) females. Thirty-nine LC patients (mean age 62.46 ± 8.70, range 47–84 years) were included in this study with 35 (89.7%) males and 4 (10.3%) females.

3.2. Metabolic differences between LC and HC groups

Principal component analysis score plot (Fig. 1A) was built based on 93 parameters containing 49 metabolites, and 44 derivatives and ratios, which were obtained from MS detection of blood metabolites of patients with LC and healthy individuals, and to reveal the discrimination trend between the 2 groups. Furthermore, PLS-DA score plot showed significant systematic metabolic differences between patients with LC and healthy individuals (Fig. 1B). The permutation test for the PLS-DA model indicated less possibility of over-fitting in this model (Fig. 1C).

3.3. The screening of significantly differential metabolites

Systematic screening for important metabolites was performed by multiple approaches. First, 30 metabolites with VIP > 1.0 were selected as important variables contributing to the classifications based on PLS-DA score plot. Second, Mann–Whitney U test or Student t test was used to evaluate significantly altered metabolites in patients with LC comparing to healthy individuals. Furthermore, false discovery rate was calculated to correct P value. A total of 22 metabolites with adjusted P value < .05 were selected between the 2 groups. Third, FC was calculated for patients with LC and healthy individuals. 35 metabolites with FC > 1.2 or < −1.2 were screened out in the comparison of LC and HC groups (Fig. 2A and B). Together, 18 metabolites were significant alterations between the 2 groups, which was shown in Figure 2C. Additionally, SAM was conducted to further screen and supervise significantly differential metabolites in patients with LC compared with healthy individuals (Fig. 3). Ultimately, 16 of these metabolites exhibited significant differences based on the screening results of volcano plots and SAM, and the changes in the 16 selected metabolites between the 2 groups were shown in Table 1. Of the 16 metabolites, levels of 14 metabolites were significantly increased; conversely, the levels of 2 metabolites were remarkably declined in LC group compared to HC group.

3.4. Prediction regression model

A stepwise logistic-regression procedure was applied to select independent parameters, which were used to build binary logistic regression model. Five parameters including arginine (Arg), proline (Pro), hexacosanoic carnitine (C26), ornithine (Orn)/citrulline (Cit), and 3-hydroxy-octadecenoylcarnitine (C18:1-OH) were screened to construct the prediction model for LC (Fig. 4). The diagnosis accuracy of the 5 selected parameters in distinguishing LC from HC was assessed independently, as shown in Table 2. The combination of 5 metabolic biomarkers displays satisfactory diagnosis ability for distinguishing patients with LC from healthy individuals. The prediction model was built as follows: Logit probability = −0.0557 + 1.6552 × Arg-
1.0578 \times \text{Pro} + 1.3351 \times \text{C26} + 1.2387 \times \text{Orn/Cit} + 1.8090 \times \text{C18:1-OH}.\) Receiver operating characteristic curve was generated to assess the diagnosis potential of these selected parameters (Fig. 5). The sensitivity and specificity for the prediction model were 0.8974 and 0.8868, respectively (Table 2). The area under receiver operating characteristic curve (AUC) for the logistic model is 0.9308 (95% CI: 0.8782–0.9834). Additionally, LOO cross-validation analysis was used to evaluate the reliability of 5 potential metabolite biomarkers. The sensitivity and specificity for the model built by the LOO cross-validation method were 0.8974 and 0.8302, respectively. The AUC for this validation model was 0.8921 (95% CI: 0.8198–0.9644). These results indicated that the metabolite panel composed of Arg, Pro, C26, Orn/Cit, and C18:1-OH has a potential to be an ideal biomarker panel to distinguish patients with LC from healthy subjects.

4. Discussion

Currently, the limitations of commonly used technologies for the detection and treatment of LC emphasize the necessity of identifying novel potential biomarkers. Metabolites could be perturbed by the specific physiological or pathological condition, and they have been served as potential biological indicators in normal and pathological and biological processes. The analysis of small-molecule metabolites can be used to identify novel biomarkers for cancer.\[19\] Hence, surveying metabolite level changes can provide a powerful approach for the early detection of HC and can aid in the diagnosis of HC.

In the present study, 2 cohorts with 92 subjects, including NC and LC groups, were formed. A combination of DBS sampling technology and direct injection MS analysis was employed to detect metabolite biomarkers. Levels of 16 metabolites showed significant differences between LC and HC groups after systematic screening. Ultimately, a biomarker panel consisting of Arg, Pro, C26, Orn/Cit, and C18:1-OH was obtained after stepwise logistic regression, and was identified and validated for the 2 groups.

Amino acid is involved in the process of protein metabolism, and plays an integral role in human function. Amino acids were disturbed by the imbalance of protein metabolism induced by host–tumor interaction as well as by metabolic requirements of tumor cell growth to specific amino acids.\[20\] Amino acid as an important part in metabolomics analysis was found to be altered in their levels in patients with cancer compared with healthy individuals.\[21\] It was used as a potential biomarker for detection of cancer,\[22\] and had a potential to improve cancer diagnosis and to detect cancer in early stage.\[22,23\] In this study, 3 amino acids and 3 ratios including Pro, Arg, Orn, Cit/Arg, Orn/Cit, and C3/methionine demonstrated significant differences in patients with LC compared with healthy subjects. Of these differential metabolites, the levels of Pro and Cit/Arg were decreased, and others were increased in patients with LC compared with healthy individuals. It is well known that activation of glycolysis or aerobic glycolysis is the major feature of cancer cell metabolism, which is called “Warburg effect.” Furthermore, deregulated anabolism/catabolism of amino acids and fatty acids have been detected as metabolic regulator of cancer cell growth.\[24,25\] Pro is a proteinogenic secondary amino acid, and its metabolism plays a critical role in cancer reprogramming.\[20\] The level of Pro was reported to be decreased in patients with head and neck cancer compared with healthy subjects,\[26\] which is consistent with our results. Proline dehydrogenase (oxidase, PRODH/POX) as the
Table 1

The differential metabolites identified by volcano plot analysis and significance analysis of microarrays in patients with laryngeal cancer compared with healthy individuals.

| No | Parameters | HC mean ± SD | LC mean ± SD | Status | P value | FDR |
|----|------------|--------------|--------------|--------|---------|-----|
| 1  | Cit/Arg    | 6.4291 ± 5.0526 | 3.6705 ± 3.9508 | ↓      | .0000   | 0.0000 |
| 2  | Pro        | 522.9987 ± 193.8309 | 385.9781 ± 230.5362 | ↓      | .0011   | 0.0073 |
| 3  | Arg        | 5.3577 ± 4.6369  | 13.2538 ± 12.5404 | ↑      | .0000   | 0.0000 |
| 4  | C26        | 0.0260 ± 0.0129  | 0.0382 ± 0.0151 | ↑      | .0000   | 0.0000 |
| 5  | C26/C20    | 0.4836 ± 0.3341  | 0.8939 ± 0.6750 | ↑      | .0000   | 0.0000 |
| 6  | C3DC       | 0.0349 ± 0.0212  | 0.0891 ± 0.0666 | ↑      | .0000   | 0.0000 |
| 7  | C18:1      | 0.4009 ± 0.1281  | 0.6203 ± 0.2561 | ↑      | .0000   | 0.0000 |
| 8  | C18:1-OH   | 0.0013 ± 0.0059  | 0.0190 ± 0.0265 | ↑      | .0000   | 0.0000 |
| 9  | C10:1      | 0.0647 ± 0.0507  | 0.1262 ± 0.0804 | ↑      | .0000   | 0.0000 |
| 10 | C3DC/C10   | 0.3868 ± 0.3153  | 0.8158 ± 0.7225 | ↑      | .0002   | 0.0019 |
| 11 | C14:2      | 0.2817 ± 0.2496  | 0.5383 ± 0.3591 | ↑      | .0002   | 0.0019 |
| 12 | C2/C0      | 0.2809 ± 0.1092  | 0.3998 ± 0.1868 | ↑      | .0008   | 0.0057 |
| 13 | Orn        | 23.2502 ± 7.8453 | 36.6597 ± 22.3176 | ↑      | .0036   | 0.0196 |
| 14 | C2         | 8.6304 ± 3.0170  | 11.6971 ± 5.9515 | ↑      | .0039   | 0.0196 |
| 15 | Orn/Cit    | 1.2032 ± 0.6647  | 1.8182 ± 1.1898 | ↑      | .0052   | 0.0242 |
| 16 | C2/Met     | 0.0560 ± 0.0254  | 0.0891 ± 0.0591 | ↑      | .0076   | 0.0337 |

Arg = arginine, C0 = free carnitine, C10:1 = decanoylcarnitine, C10 = decanoylcarnitine, C14:2 = tetradecadienoylcarnitine, C18:1 = oleylcarnitine, C18:1-OH = 3-hydroxy-octadecenoylcarnitine, C2 = acetylcarnitine, C20 = arachidic carnitine, C26 = hexacosanoic carnitine, C3 = propionylcarnitine, C3DC = malonylcarnitine, Cit = citrulline, HC = healthy control, LC = laryngeal cancer, Met = methionine, Orn = ornithine, Pro = proline.

Figure 4. Five metabolites were screened to distinguish patients with laryngeal cancer from healthy individuals by stepwise logistic regression analysis.

Table 2

Diagnostic performance of the metabolite biomarker panel for the classification of patients with laryngeal cancer and healthy individuals.

|                      | Training set | Leave one out cross-validation |
|----------------------|--------------|--------------------------------|
|                      | AUC (95% CI) | Sensitivity | Specificity | AUC (95% CI) | Sensitivity | Specificity |
| Arg                  | 0.7533 (0.6449–0.8617) | 0.6923 | 0.7925 | 0.7291 (0.6224–0.8358) | 0.6923 | 0.7736 |
| Pro                  | 0.7005 (0.5882–0.8128) | 0.6923 | 0.6792 | 0.6681 (0.5546–0.7817) | 0.6667 | 0.6604 |
| C26                  | 0.7467 (0.6463–0.8472) | 0.6154 | 0.8302 | 0.6686 (0.5551–0.7821) | 0.6154 | 0.8302 |
| Orn/Cit              | 0.6713 (0.5562–0.7863) | 0.5641 | 0.7925 | 0.6251 (0.5082–0.7419) | 0.5641 | 0.7736 |
| C18:1-OH             | 0.7937 (0.7096–0.8777) | 0.6410 | 0.9245 | 0.6207 (0.5036–0.7379) | 0.6410 | 0.9245 |
| Five-biomarker panel | 0.9308 (0.8782–0.9834) | 0.8974 | 0.8868 | 0.8921 (0.8198–0.9644) | 0.8974 | 0.8302 |

Arg = arginine, C18:1-OH = 3-hydroxy-octadecenoylcarnitine, C26 = hexacosanoic carnitine, Cit = citrulline, Orn = ornithine, Pro = proline.

Figure 4. Five metabolites were screened to distinguish patients with laryngeal cancer from healthy individuals by stepwise logistic regression analysis.
first enzyme in proline-degradative pathway is encoded by tumor protein 53-induced gene 6,[27] suggesting the possibility that the proline metabolic system may be mobilized during stress.[28] Arg as a nonessential amino acid plays a vital role in several crucial metabolic pathways.[29] Additionally, it is reported to be required for the maintenance of HeLa cell growth.[30] In this study, the level of Arg is increased, which can contribute to the decreased ratio of Cit/Arg in patients with LC compared with healthy subjects. This fact can explain that the upregulated amino acids can benefit to accelerated tumor growth. Arg is the main source of Orn, and the elevated level of Arg can influence the production of Orn, a substrate for the biosynthesis of polyamines. Polyamines are essential for cell proliferation and differentiation, and the alteration of their metabolism has been reported to be associated with cancer.[31,32] It implied that the elevated level of Orn may satisfy the requirement of polyamines for tumor cell growth.

The aberrant proliferation of tumor cells consumes more energy in the progression of malignancy, which may lead to the disordered metabolism for energy supply pathways like fatty acid beta-oxidation.[33] Beta-oxidation as the main process of fatty acid oxidation is vital for energy production, which has been found to be disturbed in various cancers.[34] Carnitine and acylcarnitines as the transporters of fatty acids into mitochondrial matrix play essential roles in the oxidative catabolism of fatty acids, and the alterations of their levels are another remarkable observation in this study. We have quantified carnitine, short-, medium-, and long-chain acylcarnitines, among them, some acylcarnitines were present in higher concentration in patients with LC compared with healthy individuals (Table 1). The levels of 2 short-chain acylcarnitines and 3 related ratios including malonylcarnitine, acetyl carnitine, malonylcarnitine/decanoylcarnitine, acetyl carnitine/free carnitine, and C3/methionine were increased in patients with LC compared with healthy individuals. Previous studies showed that the levels of carnitine and a variety of short-chain acylcarnitines were significantly elevated in cancer tissues compared with normal tissue,[11] additionally, carnitine can be used as a potential prognostic biomarker for some tumors.[35] Short-chain acylcarnitine is involved in β-oxidation after being transferred to mitochondrial matrix,[11] which suggested that the short-chain acylcarnitines were needed due to the utilization of energy source in proliferation of cancer cells. This may explain the higher levels of short-chain acylcarnitines in patients with LC than in healthy individuals. In this study, levels of 4 long-chain acylcarnitines (C26, oleylcarnitine, C18:1-OH, tetradecadienoylcarnitine) and 1 ratio (C26/arachidic carnitine) were upregulated in patients with LC compared with healthy individuals (Table 1). Long-chain acylcarnitines need specific transferases, like carnitine/acylcarnitine translocase and carnitine palmitoyl transferase (CPT) for transferring into mitochondria matrix for further hydrolysis.[10] Both CPT1A and CPT2 are rate-limiting enzymes in the beta-oxidation of long-chain fatty acids, and their expressions are closely associated with the changes of levels of acylcarnitines in patients with cancer.[36–38] A previous study revealed that the expression of CPT 2 was decreased in patients with hepatocellular cancer.[39] The increased level of long-chain acylcarnitine in this study suggested the decreased expression of CPT in patients with LC. Acylcarnitine metabolism can influence the energy production in LC, thus targeting this pathway can be regarded as a potential strategy for LC detection and treatment.

In the present study, a biomarker panel consisting of Arg, Pro, C26, Orn/Cit, and C18:1-OH was used to build prediction model by the logistic regression method for distinguishing patients with LC from healthy individuals, which showed superior prediction ability than independent metabolites (Table 2). The prediction model with AUC of 0.9308 (95% CI: 0.8782–0.9644) was also satisfactory. Furthermore, a LOO cross-validation method was used to evaluate this prediction model. The diagnostic accuracy of the LOO cross-validation model built by the biomarker panel (AUC = 0.8921, 95% CI: 0.8198–0.9644) was also satisfactory. These results highlight the diagnostic potential of the metabolite biomarker panel. Nevertheless, there were still some limitations in our study. First, this is a retrospective single-center study with a relatively small sample size. Second, LC group in this study was not divided into subgroups by cancer stages due to the small sample size. Therefore, the following research will enhance sample size, and will group patients with LC by cancer stages to discover metabolite biomarkers for the occurrence or development of LC. In addition, our results need to be confirmed with a larger sample size aimed at establishing a novel diagnostic approach.

5. Conclusion
In this study, the combination of DBS sampling technology and direct injection MS method was applied to detect metabolites, which can make MS analysis completed within few minutes. A metabolite biomarker panel based on selected metabolites including Arg, Pro, C26, Orn/Cit, and C18:1-OH was used to establish prediction model by logistic regression method, which showed higher sensitivity and specificity for distinguishing patients with LC from healthy individuals. Additionally, a LOO cross-validation method was applied to evaluate this
model, and showed satisfactory prediction performance. Therefore, it is speculated that this technology could be as an alternative method for LC prediction.

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