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ORIGINAL RESEARCH

A dried blood spot mass spectrometry metabolomic approach for rapid breast cancer detection

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Objective: Breast cancer (BC) is still a lethal threat to women worldwide. An accurate screening and diagnosis strategy performed in an easy-to-operate manner is highly warranted in clinical perspective. Besides the routinely focused protein markers, blood is full of small molecular metabolites with diverse structures and properties. This study aimed to screen metabolite markers with BC diagnosis potentials.

Methods: A dried blood spot-based direct infusion mass spectrometry (MS) metabolomic analysis was conducted for BC and non-BC differentiation. The targeted analytes included 23 amino acids and 26 acylcarnitines.

Results: Multivariate analysis screened out 21 BC-related metabolites in the blood. Regression analysis generated a diagnosis model consisting of parameters Pip, Asn, Pro, C14:1/C16, Phe/Tyr, and Gly/Ala. Tested with another set of BC and non-BC samples, this model showed a sensitivity of 92.2% and a specificity of 84.4%. Compared to the routinely used protein markers, this model exhibited distinct advantage with its higher sensitivity.

Conclusion: Blood metabolites screening is a more plausible approach for BC detection. Furthermore, this direct MS analysis could be finished within few minutes, which means that its throughput is higher than the currently used imaging techniques.

Keywords: breast cancer, metabolomics, dried blood spot testing

Introduction

Breast cancer (BC) is the leading cause of cancer-related deaths in women. Although there are many newly emerged screening and therapeutic measures, the morbidity and mortality are still not satisfactorily controlled, posing a great challenge to public health.[1] Annually, it is estimated that ~1.3 million new BC cases are diagnosed worldwide.[2]

A growing body of evidence has demonstrated that BC patients' outcome is intensely influenced by cancer stage at the point of diagnosis. Early-stage patients have higher 5-year survival rate than those diagnosed at later stage.[3] Thus, effective BC screening plays key roles in improving survival rates and prognosis. The discovery of BRCA1/2, ERBB2, ESR1/ER, and relevant genes strengthens our ability to discriminate, screen, and treat BC. Unfortunately, the cost and availability of the relevant facilities prevent routine application of genetic screening, especially in the undeveloped countries. Additionally, utility of solely genetic information for early BC detection is not fully convincing.[4]

Currently, BC screening largely relies on radiologic and serum protein marker detection strategies.[5,6] Magnetic resonance imaging and computed tomography are relatively reliable tactics that help BC diagnosis clinically, but they are not cost-effective and are not easily accessed by citizens living in the undeveloped regions.[7] Although mammography checking has claimed to reduce BC-related deaths from 13% to 25%, it

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is at the expense of ~30% overdiagnosis in addition to the risk of large-dose radiation exposure.[8] In addition, this screening needs the presence of potential patients in the whole checking process, making it a time-consuming operation.

Blood tumor marker detection has been widely advocated for BC screening, diagnosis, and prognostic prediction. The widely used markers include, but are not limited to, carcinoembryonic antigen (CEA), cancer antigens (CA 27.29, CA 15.3), tissue polypeptide-specific antigen, and tissue polypeptide antigen (TPA). However, these markers lack desired specificity and sensitivity, underscoring the urgent need for alternative simple, accurate, and easy-to-perform screening approaches.[9]

Cancer cells show distinct metabolic characteristics compared to their normal counterparts. Although incomplete, our understanding of metabolic remodeling in cancer cells has been largely reinforced since the discovery of the so-called Warburg effect.[10–13] The realization of quantitative and qualitative analysis of as many metabolites as possible in a certain system (cell, tissue, or biofluid) in a single run gives birth to the science of metabolomics. Since this conception was firstly coined, metabolomics has been widely used in different aspects of life sciences. The benefited fields include, but are not limited to, disease stratification, biomarker discovery, drug side effect evaluation, and unknown gene function elucidation.[14–17]

Currently prevailing metabolomic techniques mainly include chromatography–mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy.[18] The former employs chromatography to separate different metabolites firstly, and then detects the eluted analytes by the MS system hyphenated to it. This mechanism means that the whole analysis process will need more time, implying that its throughput will be limited to some extent.[14] The latter can directly analyze different raw samples, rendering it a high-throughput feature but usually with low sensitivity.[19]

In BC metabolomic analysis, utilizing gas chromatography–MS system, beta-alanine, 2-hydroyglutarate, glutamate, and xanthine were found increased and glutamine was found decreased in the estrogen receptor-positive BC tissues.[20] Using an NMR-based serum profiling tactic, histidine, glycerol, acetoacetate, pyruvate, mannose, phenylalanine, and glutamate were reported to be valuable in discriminating patients with metastatic or localized BC.[21] Unlike blood and tissue metabolomics, urine samples had been explored for screening potential metabolite markers aiding BC diagnosis.[22]

The entry of MS technology into clinical laboratory was earlier than NMR. The maintenance of MS is easier and cheaper than that of NMR in many aspects. MS-based newborn screening (NS) has been applied for decades.[23] What should be mentioned specifically is that NS usually employs dried blood spot (DBS) samples collected by heel or finger puncture to simplify the process. Compared to the traditional venous blood samples, the advantages of DBS are that volume of blood taken is much less, they do not need expensive vacuum sample tubes, and they are easy to transport and store. Furthermore, the utilization of direct infusion MS in NS greatly improves the analysis throughput.[24]

Since the DBS-based MS analysis technique can be used for newborn metabolic disorders screening, it might also be useful in tumor metabolite marker screening. In light with this, a DBS-based metabolomic study was performed by using direct infusion MS/MS analysis of BC and the control samples in this study. The quantified metabolites include 23 amino acids and 26 acylcarnitines (Tables S1 and S2), which are commonly encountered in NS. Some ratios based on the above metabolites were also calculated to enrich the analysis information (Table S3). A regression model was then constructed by using parameters that were differentially changed between the two groups. By employing another set of samples, diagnosis ability was evaluated in view of sensitivity and specificity. The purpose of this study was to answer if the DBS-based direct infusion MS technique could be used to facilitate BC screening and diagnosis.

Materials and methods

Sample information

DBS samples from 258 newly diagnosed BC patients and 159 benign mammary gland disease control patients (including 78 healthy people) were collected from The First Affiliated Hospital of Liaoning Medical University. The average ages of BC patients and the controls were 60.4 years (44–80) and 58.7 (42–83), respectively. Statistical analysis showed no age difference between the two groups (P=0.351, Student's t-test). The study was approved by the Ethics Committee of The First Affiliated Hospital of Liaoning Medical University. Before DBS collection, written informed consents were acquired from the participants. Of the two groups, metabolomic data from randomly selected 207 patients and 127 age-matched controls (~80% of the total samples) were used as training set to establish regression diagnosis model. The remaining

20% samples were used to evaluate the applicability of the constructed model. All the specimens were fasting blood samples.

Chemicals

Acetonitrile (high-performance liquid chromatography-grade) and high-purity water were obtained from Thermo Fisher (Waltham, MA, USA). 1-Butanol and acetyl chloride were commercially acquired from Sigma-Aldrich (St Louis, MO, USA). Isotope-labeled internal standards of 12 amino acids (NSK-A) and eight acylcarnitine (NSK-B) from Cambridge Isotope Laboratories (Tewksbury, MA, USA) were used for absolute quantification purpose. All the standards were mixed and dissolved in 2 mL pure methanol and stored at 4°C. Working solution was prepared through 100-fold dilution for metabolite extraction. Amino acids and carnitines quality control (QC) standards were provided by Chromsystems (Grafelfing, Germany). The QC samples were treated as real samples and processed according to the provided instructions to ensure the analysis stability.

Sample preparation

Except mentioned specifically, all the tests were conducted at room temperature. A 3 mm (diameter) disc was punched from each DBS paper. The discs were placed into the Millipore MultiScreen HV 96-well plate (Millipore, Billerica, MA, USA) for metabolite extraction. Briefly, every 100 µL working solution was added into each well containing a DBS disc. After 20-minute gentle shaking, the plates were centrifuged at 1,500× *g* for 2 minutes. The filtrate was collected using new flat-bottom 96-well plates. For each plate, four randomly selected blank wells were added with two low-level and two high-level QC control solutions individually. The QC and filtrate solutions were dried by pure nitrogen gas flow at 50°C. Dried samples were derivatized at 65°C for 20 minutes using 60 µL acetyl chloride/1-butanol (10:90, v/v) mixture. The derivatized samples were dried again as mentioned earlier. For metabolomic analysis, each dried sample was dissolved in 100 µL fresh mobile phase solution.

Metabolomic analysis

The direct injection MS metabolomic analysis was conducted by using an AB Sciex 4000 QTrap system (AB Sciex, Framingham, MA, USA). The equipped ion source was electrospray ionization source. All the analytes were scanned under positive mode, and the detailed scan parameters are given in Tables S1 and S2.

For each run, every 20 µL sample was injected. The mobile phase was 80% acetonitrile aqueous solution. The initial flow rate was 0.2 mL/min. Subsequently, the flow rate was reduced to 0.01 mL/min within 0.08 minute, kept constant until 1.5 minutes, returned to 0.2 mL/min within 0.01 minute, and held constant for another 0.5 minute. The ion spray voltage was 4.5 kV. Curtain gas pressure was set at 20 psi. A 35 psi pressure was applied to ion source gas 1 and gas 2. The auxiliary gas temperature was maintained at 350°C.

Analyst v1.6.0 software (AB Sciex) was used for system control and data collection. ChemoView 2.0.2 (AB Sciex) was used for data preprocessing. Partial least squares-discriminant analysis (PLS-DA) was performed by using SIMCA-P v12.0 (Umetrics, Umeå, Sweden). For establishment of BC diagnosis model, binary logistic regression was conducted by using MINITAB v16.0 (Minitab, State College, PA, USA). The diagnostic ability was evaluated by area under the receiver operating characteristic curve. The remaining 20% samples of each group were used for diagnosis ability appraisal.

Results

The two groups showed distinct metabolomic difference

To ensure the method robustness, the QC sample data were firstly evaluated. Detected values from the QC samples all fell into the recommended ranges (±2 standard deviation), indicating the satisfactory performance of the analysis (data not shown).

For the real samples, a total of 49 metabolites and 22 ratios were detected and calculated for each sample. Using those parameters, a PLS-DA model was established and it showed a clear separation trend between the BC and control groups (Figure 1A). To test if model overfitting has occurred, a permutation test based on 100 iterations was conducted to appraise fitness of the original model against the randomly permuted models.[25] This operation demonstrated that there was less possibility that the overfitting has occurred in the PLS-DA model (Figure 1B). Thus, the analysis implied that there were really some parameters showing distinct levels between the two groups.[25]

Differential parameter selection

Using randomly selected 80% of the BC and control samples, a multivariate analysis[26] was carried out to lock potential parameters that had statistic difference between the two groups. It was found that 22 parameters decreased in the

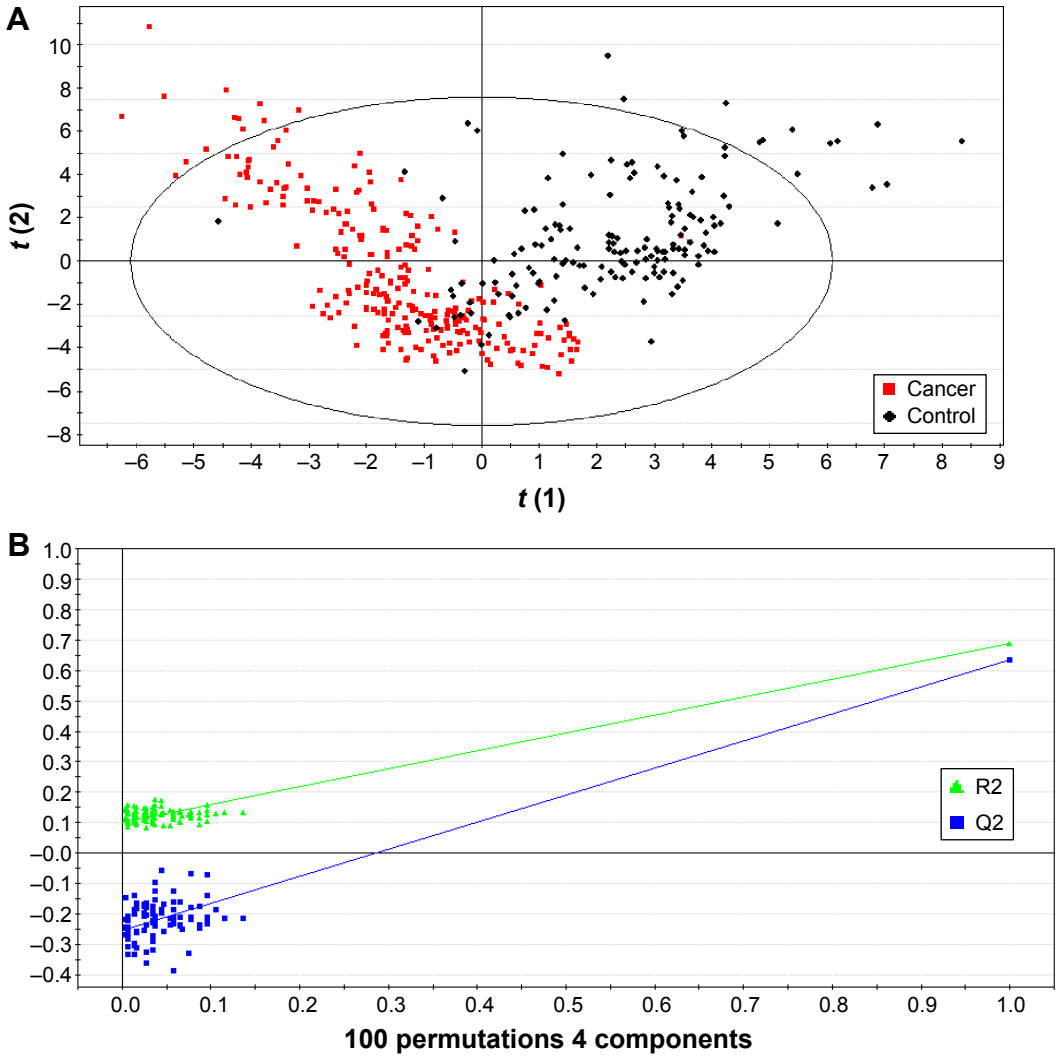

Figure 1 Partial least squares-discriminant analysis of the metabolomic data.
Notes: (**A**) Scores plot showing the discrimination between BC and non-BC samples. (**B**) A 100-time permutation test for validating the corresponding model. The *Y*-axis intercepts were R^2 (0.0, 0.101) and Q2 (0.0, –0.254).
Abbreviation: BC, breast cancer.

BC group and 13 parameters increased in the BC group (Figure 2). These variables were further reevaluated by *t*-test, and those of *P*-values <0.05 were kept. Finally, 21 parameters were verified to be significantly different between the two groups with only C2, C3, and Tyr increased in the BC group (Table 1).

Diagnostic regression equation

In order to test if the 21 differential parameters could be used for BC diagnosis purpose, a binary logistic regression analysis was conducted. The final equation contained only three amino acids and three calculated ratios, Pip, Asn, Pro, C14:1/C16, Phe/Tyr, and Gly/Ala (Figure 3). Receiver operating characteristic evaluating the diagnosis model gave a sensitivity and specificity of 90.3% and 87.4%, respectively, when the cutoff was set to 4.8754 (Figure 4). Area under the curve was 0.944. Further tested by using each remaining 20% samples of each group, the model gave a diagnosis sensitivity of 92.16% and a specificity of 84.38%.

Discussion

One of the major challenges for successful diagnosis and treatment of BC is the lack of reliable molecular predictors. Over the past decades, there have been a rapidly growing number of metabolomic researches aimed at finding biomarkers that could be used to aid BC diagnosis, evaluate response to therapy, and provide treatment guidance.[27,28]

Although tumor cells show distinct metabolic features, they really share the same metabolic pathways and metabolites with their normal counterparts. Thus, we performed a

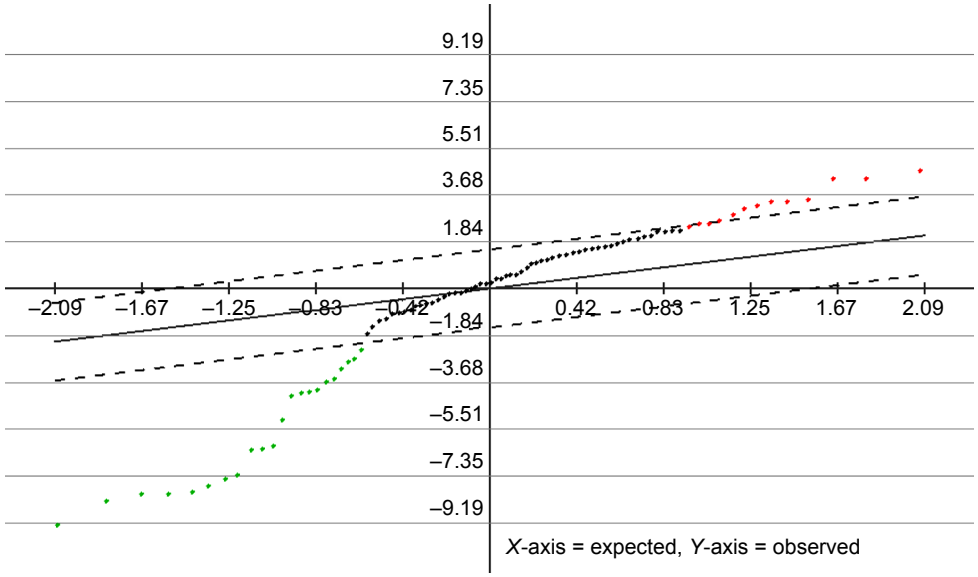

Figure 2 SAM analysis results with the false discovery rate set to zero.
Notes: Points (metabolites or ratios) above (elevated in BC group) or under (decreased in BC group) the dashed lines were those changed significantly.
Abbreviations: BC, breast cancer; SAM, significance analysis of microarrays.

DBS-based metabolomic assay aimed at amino acids and acylcarnitines, attempted to find valuable clues to help BC diagnosis.

Amino acids are the basic building blocks for nearly all cell types. In the context of cancer metabolomics, many amino acids have been demonstrated to provide valuable clues for studying pathogenesis and to act as potential indicators for diverse malignancies.[29] Specific plasma amino acid changes have been reported in patients suffering from breast, lung, and head and neck cancers through metabolomic analysis.[30,31]

Table 1 Differential parameters between BC and control groups identified using the training set data

No	Parameters	Status*	*P*-value	Control mean ± SD	BC mean ± SD
1	C3	↑	0.000	1.4±0.7	2.1±1.3
2	Tyr	↑	0.001	42.6±12.5	51.0±16.8
3	C2	↑	0.000	13.1±8.0	19.5±10.8
4	Cys	↓	0.000	1.2±1.0	0.44±0.45
5	Pro	↓	0.000	308.2±175.2	194.7±82.5
6	Asn	↓	0.000	71.0±28.1	47.6±25.1
7	Pip	↓	0.000	352.1±452.8	97.0±97.3
8	Hcy	↓	0.000	6.9±1.6	5.8±0.9
9	Trp	↓	0.000	47.1±21.1	28.8±20.7
10	C14:2	↓	0.000	5.1±7.7	0.9±2.0
11	C10:2/C10	↓	0.000	5.2±3.9	2.4±2.6
12	C10:2	↓	0.000	0.3±0.2	0.2±0.1
13	Phe/Tyr	↓	0.000	1.2±0.3	1.0±0.2
14	Cit/Arg	↓	0.002	6.1±5.5	3.3±3.3
15	Lys	↓	0.000	72.2±121.9	25.5±40.9
16	C18:1-OH	↓	0.001	2.7±5.5	1.0±2.0
17	His	↓	0.000	57.3±134.8	19.0±23.9
18	Tyr/Cit	↓	0.011	0.9±0.4	0.7±0.3
19	C14:1/C16	↓	0.000	0.9±0.5	0.2±0.3
20	C18-OH	↓	0.002	1.6±4.6	0.6±1.3
21	Gly/Ala	↓	0.001	157.1±69.4	154.3±48.1

Note: *Compared to the control group.
Abbreviations: BC, breast cancer; SD, standard deviation.

Cascino et al found that Orn, Glu, and Trp increased in the patient plasma.[30] Miyagi et al demonstrated that Gln, His, Trp, Tyr, and Phe decreased in the plasma, whereas Gly, Ala, Pro, and Thr increased.[32] In this study, only Tyr increased in BC blood, but Cys, Pro, Asn, Pip, Hcy, Trp, Lys, and His decreased (Table 1). This discrepancy might be due to the fact that only in this study, the control group included the benign mammary gland diseases and the healthy people simultaneously. Except the disagreement among these studies, Tyr deficiency had been demonstrated to result in BC cell growth arrest.[33] Thus, increased blood Tyr might do favor to BC. Of note, blood Tyr is affected by diet. It cannot be de novo synthesized by human body. It is also not easy to exclude the possibility that increased Tyr might be the metabolic adaptation to tumor state.

Except Tyr, there were eight decreased amino acids in the BC blood. It was most likely that such phenomena were the result of excessive consumption of amino acids by BC tissues. As what has been well accepted, tumor cells need more amino acids to sustain their uncontrolled growth.[34]

Carnitine plays key roles in fatty acids catabolism. It acts as a shutter to bring fatty acids into mitochondria for

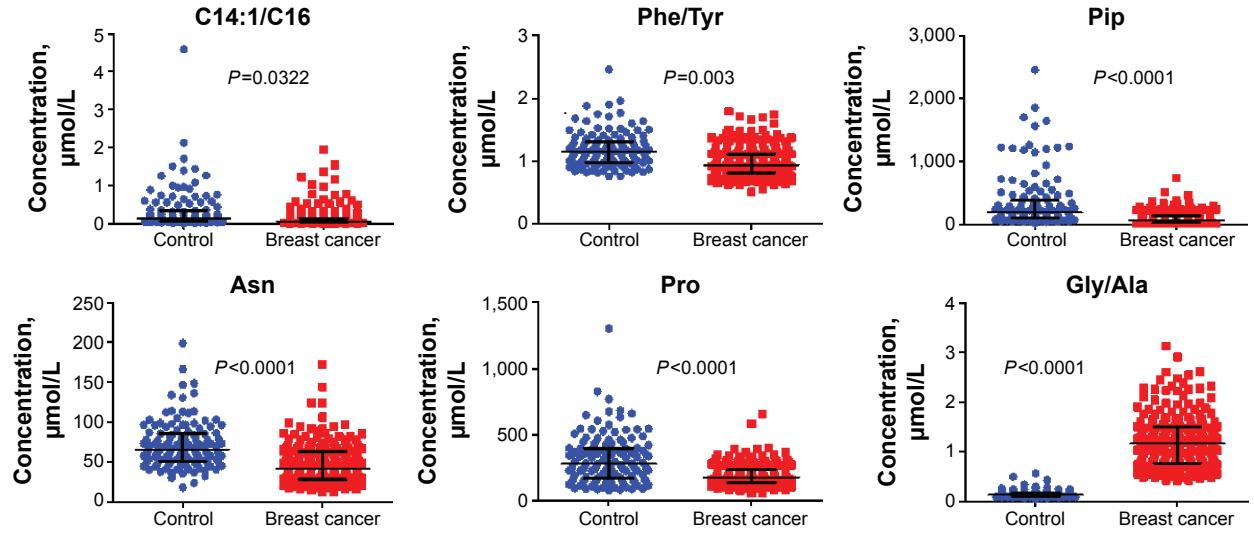

Figure 3 Levels of six metabolites included in the regression model.

oxidation. A clear trend in this study was that short-chain carnitine increased in BC plasma (Table 1). In a study of myeloma, C2 carnitine was identified as a potential biomarker to indicate disease activity and relapse.[35] Increased C2 carnitine was also reported in the urine of patients suffering from hepatocellular carcinoma.[36] Also in hepatocellular carcinoma, C3 carnitine was found at high level in cancer tissues.[37] Although there was no evidence to correlate short-chain carnitines with BC, they were more likely to be potential markers to indicate the occurrence of many malignancies.

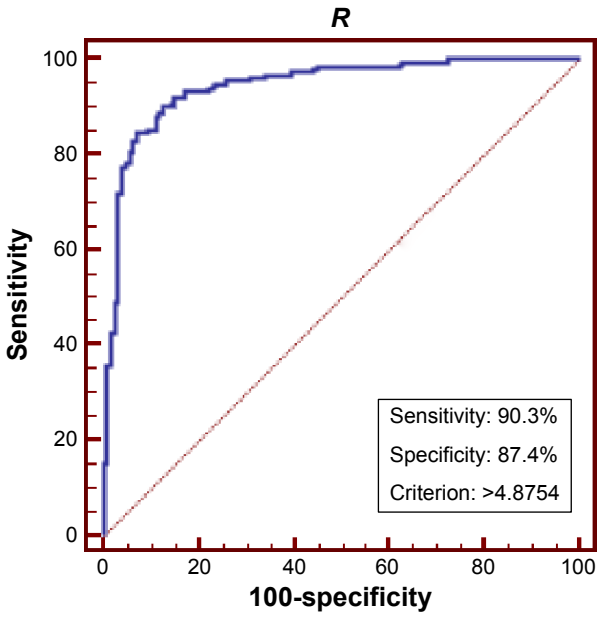

Figure 4 ROC based on the regression model.
Note: Model equation was $y = -C14:1/C16 \times 4.24 - Phe/Tyr \times 3.32 - Pip \times 0.01 - Asn \times 0.05 - Pro \times 0.01 - Gly/Ala \times 3.11 + 14.91$.
Abbreviations: ROC, receiver operating characteristic; R, regression result.

In order to explore the utility of the differentially expressed metabolites, a regression model was constructed by using parameters listed in Table 1. The result showed that combined use of six parameters could appropriately differentiate BC from non-BC samples (Figures 3 and 4). Among them, only one ratio, the carnitines C14:1/C16, was included. Increased C14:1/C16 acylcarnitine ratio was closely linked to impaired mitochondria fatty acid beta-oxidation,[38] coinciding with the fact that mitochondrial impairment had been demonstrated in BC cells.[39]

In the regression model, Pip, Asn, and Pro were the only included free amino acids. Their levels were all decreased. We speculated that the decrease was more likely due to the excessive consumption by tumor cells. Besides the free amino acids, elevated Phe/Tyr and decreased Gly/Ala were closely related to the differentiation of BC and non-BC (Figure 3). High ratio of Phe/Tyr had been described in malignant histiocytosis and some other cancerous diseases.[40,41] As of the decreased Gly/Ala ratio, it was most possible owing to the fact that rapid proliferating tumor cells need more Gly.[34]

Although the diagnosis specificity (87.4%) of the regression model was not comparable with that of CA 15.3 (93%–95%), CEA (45%–95%), and TPA (~81%), the sensitivity (90.3%) was superior to that of CA 15.3 (44%–64%), CEA (~45%), and TPA (~67%).[42,43] Thus, for BC screening purpose, the DBS-based MS technique was a promising tool for early BC discovery.

Conclusion

In summary, a proper diagnostic model with only six blood parameters was established and could be utilized to

discriminate BC from non-BC. It could be expected that the DBS-based MS strategy was a promising alternative for BC screening because of its higher sensitivity. Additionally, the whole MS analysis could be completed within few minutes. Unfortunately, the traditional plasma protein tumor markers were not simultaneously measured for these specimens; therefore, the diagnosis ability of combined use of traditional markers and the metabolite markers could not be evaluated. Further analysis should be addressed to the relevant topics to ascertain the exact value of DBS-based MS metabolomic analysis in BC screening and diagnosis.

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Disclosure

The authors declare no conflicts of interest in this work.

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Supplementary materials

Table S1 The amino acids detected, corresponding scan modes, equipment settings, and quantification IS used in the study

No	Abbreviation	Full name	Scan type	Loss (Da)	Start–stop (Da)	Fragment transition	DP (V)	EP (V)	CE (V)	CXP (V)	IS	IS (μM)
1	Ala	Alanine	Neutral loss	102.1	130–280		40	10	19	3	d4-Ala	2.5
2	Arg	Arginine	MRM			231.2→70.0	55	10	41	2	d5-Arg	2.5
3	Asn	Asparagine	Neutral loss	102.1	130–280		40	10	19	3	d3-Leu	2.5
4	Asp	Aspartate	Neutral loss	102.1	130–280		40	10	19	3	d3-Asp	2.5
5	Cit	Citrulline	MRM			232.2→113.1	44	10	25	2	d2-Cit	2.5
6	Cys	Cysteine	Neutral loss	102.1	130–280		40	10	19	3	d8-Val	2.5
7	Gln	Glutamine	Neutral loss	102.1	130–280		40	10	19	3	d3-Met	2.5
8	Glu	Glutamic	Neutral loss	102.1	130–280		40	10	19	3	d3-Glu	2.5
9	Gly	Glycine	MRM			132.1→76.0	36	10	14	2	15N13C-Gly	12.5
10	Hcy	Homocysteine	Neutral loss	102.1	130–280		40	10	19	3	d3-Leu	2.5
11	His	Histidine	Neutral loss	102.1	130–280		40	10	19	3	d3-Met	2.5
12	Leu	Leucine	Neutral loss	102.1	130–280		40	10	19	3	d3-Leu	2.5
13	Lys	Lysine	Neutral loss	102.1	130–280		40	10	19	3	d3-Met	2.5
14	Met	Methionine	Neutral loss	102.1	130–280		40	10	19	3	d3-Met	2.5
15	Orn	Ornithine	MRM			189.2→70.1	37	10	34	2	d2-Orn	2.5
16	Phe	Phenylalanine	Neutral loss	102.1	130–280		40	10	19	3	d6-Phe	2.5
17	Pip	Piperamide	Neutral loss	102.1	130–280		40	10	19	3	d8-Val	2.5
18	Pro	Proline	Neutral loss	102.1	130–280		40	10	19	3	d8-Val	2.5
19	Ser	Serine	Neutral loss	102.1	130–280		40	10	19	3	d4-Ala	2.5
20	Thr	Threonine	Neutral loss	102.1	130–280		40	10	19	3	d8-Val	2.5
21	Trp	Tryptophan	Neutral loss	102.1	130–280		40	10	19	3	d3-Glu	2.5
22	Tyr	Tyrosine	Neutral loss	102.1	130–280		40	10	19	3	13C6-Tyr	2.5
23	Val	Valine	Neutral loss	102.1	130–280		40	10	19	3	d8-Val	2.5

Abbreviations: IS, internal standard; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential; MRM, multiple reaction monitoring.

Table S2 The precursor scan mode-detected carnitines, corresponding equipment settings, and quantification IS used in the study

No	Abbreviation	Full name	Start–stop (Da)	Precursor (*m/z*)	DP (V)	EP (V)	CE (V)	CXP (V)	IS	IS (μM)
1	C0	Free carnitine	210–610	85.1	40–75	10	35–55	3	d9-C0	0.76
2	C2	Acetylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C2	0.19
3	C3	Propionylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C3	0.04
4	C4	Butyrylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C4	0.04
5	C4OH	3-Hydroxylbutyrylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C5	0.04
6	C4DC	Succinyl-/methylmalonylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C8	0.04
7	C5	Cisovalerylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C5	0.04
8	C5-OH	3-Hydroxyisovalerylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C5	0.04
9	C5DC	Glutarylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C8	0.04
10	C5:1	Tiglylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C5	0.04
11	C6	Hexanoylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C5	0.04
12	C8	Octanoylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C8	0.04
13	C10	Decanoylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C8	0.04
14	C12	Lauroylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C14	0.04
15	C14	Myristoylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C14	0.04
16	C14-OH	3-Hydroxyl-tetradecanoylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C14	0.04
17	C14DC	Tetradecanoyldiacylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
18	C14:1	Tetradecenoylcarnitine	210–610	85.1	40–75	10	35–55	3	d9-C14	0.04
19	C16	Palmitoylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
20	C16-OH	3-Hydroxypalmitoylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
21	C16:1-OH	3-Hydroxypalmitoleylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
22	C18	Octadecanoylcarnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
23	C20	Arachidic carnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
24	C22	Behenic carnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
25	C24	Tetracosanoic carnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08
26	C26	Hexacosanoic carnitine	210–610	85.1	40–75	10	35–55	3	d3-C16	0.08

Abbreviations: IS, internal standard; DP, declustering potential; EP, entrance potential; CE, collision energy; CXP, collision cell exit potential; m/z, mass/charge ratio.

Table S3 Parameters derived from the quantified metabolites

No	Name	No	Name
1	Arg/Orn	23	C5DC/C16
2	Cit/Arg	24	C8/C2
3	Gly/Ala	25	C8/C10
4	Met/Leu	26	C16-OH/C16
5	Met/Phe	27	C26/C20
6	Orn/Cit	28	C14:1/C16
7	Phe/Tyr	29	C3DC
8	Tyr/Cit	30	C3DC/C10
9	Val/Phe	31	C18:1
10	C2/C0	32	C18-OH
11	C3/C0	33	C18:1-OH
12	C3/C2	34	C10:1
13	C3/C16	35	C10:2
14	C4/C2	36	C14:2
15	C4/C3	37	C18:2
16	C4/C8	38	C10:2/C10
17	C5/C0	39	C6DC
18	C5/C2	40	C5DC/C8
19	C5/C3	41	(0+2+3+16+18:1)/Cit
20	C5-OH/C8	42	(C16+C18)/C0
21	C5-OH/C0	43	C0/(C16+C18)
22	C5DC/C5-OH	44	C3/Met