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Original Article

Nontargeted metabolite profiling evaluation in patients with myelodysplastic syndrome based on LC-QTOF-MS

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Abstract: The present study aimed to explore the abnormal metabolites of patients with myelodysplastic syndrome (MDS) using liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS). Both serum and urine samples from 20 patients with MDS (MDS group) and 20 healthy volunteers (normal control, NC group) were collected, and the metabolite profiles were analyzed systematically using LC-QTOF-MS. Untargeted substantial differences were detected between the metabolite signatures of the MDS and NC groups using partial least-squares discriminant analysis. A total of 12 metabolites in the amino acid and nucleotide metabolic pathways presented abnormal levels in the serum. Abnormalities were also detected in 7 metabolites in the urine. Thus, these identified abnormal metabolites suggested a substantial metabolite disorder in patients with MDS, which would be valuable in further investigation and diagnosis of MDS.

Keywords: Metabolomics, myelodysplastic syndrome, LC-QTOF-MS, amino acid, nucleotide

Introduction

Myelodysplastic syndrome (MDS) is a malignant stem-cell disease characterized by ineffective and dysplastic hematopoiesis [1]. The incidence and prevalence of the disease is about 4/100,000 and 7/100,000 individuals/year, respectively [2]. Presently, the diagnosis of MDS relies on the morphological assessment of the peripheral blood and bone marrow, conventional cytogenetics, and exclusion of secondary causes of dysplasia [3]. However, diagnosis can be challenging due to the heavy reliance on the morphological assessment of MDS [4]. Blood tests usually do not establish the presence of the disease unambiguously during the early stages or remission, and examination of the smears of bone marrow aspirates, and immunologic methods are not adequate in early diagnosis because the symptoms are often vague and unspecific [2, 3]. Therefore, identifying biomarkers for early, noninvasive detection of MDS could be a lifesaving intervention.

Nontargeted metabolite profiling or metabolomics is a hypothesis-free study approach that finds the differences in metabolite profiles among study subjects, thereby identifying the novel small-sized molecular biomarkers for disease progression or prevention [5]. A holistic view of the metabolites can be obtained by metabolomics methods with respect to amino acids, carbohydrates, lipids, peptides, nucleic acids, and vitamins in specific cells, tissues, or body fluids [6]. A recent clinical study showed that 9 metabolites were altered significantly in 20 serum samples from 10 patients with acute myeloid leukemia (AML) [7]. Other studies also confirmed the systemic supply disorder of nutrients, especially amino acid and fatty acid metabolism in AML cells [8, 9]. In a previous study, we identified multiple metabolite abnormalities in the serum of patients with bone marrow failure syndrome (BMFS) [10]. Thus, metabolomics is an emerging approach to studying the metabolism of systemic hematological diseases, which will be helpful in identifying the specific metabolites as diagnostic or prognostic markers of these diseases [11].

MDS is a specific form of acquired BMFS [12, 13], characterized by disrupted differentiation and maturation and altered bone marrow stroma [14]. Moreover, about 25% of all patients with MDS eventually evolve into having AML in subsequent disease development [15, 16]. Although metabolomics has been widely used to investigate BMFS [10, 13] and AML [17, 18], there are few reports on the metabolite profiles of MDS throughout the world. In this study, we assessed the presence of abnormal metabolite profiles in the serum and urine of MDS patients and further evaluated the potentially disordered metabolite pathway in patients with MDS.

Patients and methods

Patients

Between December 2017 and November 2019, 20 patients with MDS were treated at the Hematology ward of the Second Affiliated Hospital of Qiqihar Medical College. The study was approved by the Qiqihar Medical College Ethics Committee, according to the Declaration of Helsinki. All individual participants provided informed consent before participation in this study. All patients were diagnosed for the first time according to the 2016 World Health Organization (WHO) classification scheme for MDS [3]. The cohort was comprised of 9 males and 11 females. In addition, 20 healthy individuals, consisting of 12 males and 8 females, were recruited as normal controls (NC). Serum and urine samples were collected in accordance with the approved guidelines. Briefly, after overnight fasting (for at least 10 h), blood samples were collected by venipuncture in BD tubes. Serum was collected by resting the blood samples at 4°C for 2-3 h, followed by centrifugation at 3,000 rpm, 4°C for 20 min. Then, the serum samples were divided into two parts. One was subjected to laboratory measurements, including white blood cell count (WBC), neutrophil count (Neu), hemoglobin level (HGB), and platelet count (PLT) on a Hitachi 7600 Clinical Chemistry Analyzer (Hitachi, Japan). The other part of the specimine was frozen at -80°C for further metabolic analysis. Similarly, clean morning urine samples were collected from all participants and frozen at -80°C for further testing.

Sample preparation

The samples for the analysis of the two groups were prepared as described previously [10]. Briefly, serum and urine samples were thawed at 4°C for 50 min and vortexed for 30 s. An equivalent of 100 μL sample was mixed with 400 μL of acetonitrile and vortexed for 2 min. The supernatant was collected by centrifugation at 14,000×g, 4°C for 15 min. A volume of 100 μL was transferred into another tube and dried on a heat block at 35°C under a gentle stream of nitrogen gas. The pellet was dissolved in 100 μL of acetonitrile/water (1:3, v/v) and vortexed for 1 min. An equivalent of 100 μL supernatant, collected by centrifugation at 14,000×g, 4°C for 15 min, was transferred to the autosampler vials and injected into liquid chromatography-hybrid quadrupole time-of-flight mass spectrometry (LC-QTOF-MS) for analysis. To ensure the stability and repeatability of the UPLC/MS system, pooled quality control (QC) samples were prepared by mixing equal amounts of supernatant samples from the two groups.

Chromatography

A volume of 10 μL pre-treated samples was injected on a 3.0 × 100 mm (1.8 μm) ZORBAX SB-C18 column (Agilent Technologies, USA) for RRLC. The MS chromatography was obtained by electrospray ionization (ESI) source under two sets of mobile phases, positive-ion (ESI+) and negative-ion (ESI-) modes. The mobile phase for ESI+ was a mixture of acetonitrile containing 0.1% formic acid (A) and water containing 0.1% formic acid (B). The mobile phase for ESI- was a mixture of acetonitrile (A) and water (B). For both modes, a linear mobile phase gradient was used as follows: 2% A, held for 1 min; 1-18 min, increased to 98% A; 18-21 min, held at 98% A; 21-21.1 min, decreased to 2% A and 21.1-28 min, held at 2% A. The mobile phase flow rate was 0.3 mL/min at 40°C [19].

Mass spectrometry

MS was performed on an Agilent 6530-QTOF (Agilent Technologies) equipped with ESI+ and ESI- modes. The capillary voltage was set at 4.0 kV for the ESI+ mode and 3.5 kV for the ESI- mode. The desolution gas adopted nitrogen at a flow rate of 10 L/min at 350°C. Then, cen-

Table 1. Descriptive characteristics of MDS and NC groups in age, sex, and routine blood tests

Characteristic	MDS group (n = 20)	NC group (n = 20)	t value	*P* value
Gender (M/F)	9/11	12/8		
Age	63.1 ± 14.65	61.8 ± 17.59	0.25	P = 0.08
WBC	1.73 ± 0.94	6.79 ± 1.16	15.18	P<0.001
Neu	0.74 ± 0.60	4.36 ± 1.21	12.00	P<0.001
HGB	68.75 ± 22.56	140.3 ± 12.2	12.48	P<0.001
PLT	61.8 ± 65.74	217.7 ± 44.18	8.80	P<0.001

Abbreviations: WBC, white blood cell count; Neu, neutrophil count; HGB, hemoglobin level; PLT, platelet count.

troid data were collected in full scan mode from 50-1,000 m/z.

Data preprocessing and annotation

The data were input into a Mass-Hunter Qualitative Analysis Software (Agilent Technologies) to obtain mz data-format files, which were then imported to the XCMS package in R for preprocessing [xcmsSet (fwhm = 10); group (minfrac = 0.5, bw = 30)]. The results included the retention time, m/z values, and peak intensity. CAMERA in R was used for annotation of isotope peaks, adducts, and fragments in the peak lists [20]. After data processing, 1,142 ions were obtained from serum and 493 from urine in the ESI+ mode, while 525 ions were detected in serum and 401 in urine in the ESI- mode. All data were subjected to further statistical analysis.

Statistical analysis

First, the grouping trend and the abnormal values were detected by principal component analysis (PCA) method [21]. Subsequently, the metabolic patterns of the two groups (MDS and NC) were investigated using partial least squares discriminant analysis (PLS-DA) [22, 23]. The variable importance in the projection (VIP) was calculated based on PLS-DA model. The significance of metabolites was determined using non-parametric Wilcoxon testing, and the corresponding false-discovery rate (FDR) value was corrected for multiple test results. The criteria of metabolic markers were as follows: VIP>1 and FDR<0.05. The 100-iteration replacement test was carried out to determine the match level of the PLA-FDA model. The PCA and PLS-DA tests were carried out using SIMCA-P software, while the other statistical analyses were conducted on the R platform [24]. In addition, Student's *t*-test was employed to evaluate the clinical characteristics (age, WBC, Neu, HGB, and PLT) in the MDS and NC groups using Prism 6.0 software (GraphPad, USA). All data were reported as the mean ± the standard deviation. P<0.05 or 0.01 was considered statistically significant.

Results

Clinical characteristics of subjects

A total of 20 patients with MDS (F/M = 9/11, average age = 68.4 ± 23.5 years) comprised the MDS group and 20 healthy individuals (female: male ratio = 12:8, average age = 62.25 ± 27.23 years) were enrolled in the NC group. The serum characteristics, including the WBC, Neu, HGB, and PLT count, were compared between the two groups (**Table 1**). Compared to the NC group, the WBC, Neu, HGB, and PLT values were significantly lower in the MDS group (P<0.01). These changes also match the clinical diagnostic criteria of MDS.

Quality control evaluation

Figure 1 shows that the PCA on serum and urine samples evaluated the dispersion degree of our metabolic profiling. The results revealed that the QC samples are tightly clustered in the PCA score plots, indicating the robustness of our metabolic profiling platform.

PLS-DA model analysis

The differences in the serum and urine samples were determined by a supervised PLS-DA modeling between the two groups, as shown in **Figures 2** and **3**. The current results showed a clear distinction between the NC and MDS groups in the ESI+ and ESI- modes. The PLS-DA models consisted of two predictive components in the ESI+ mode (R2Ycum = 0.941, Q2cum = 0.917) and two components in the ESI- mode (R2Ycum = 0.902, Q2cum = 0.855) in serum. The PLS-DA models contained two predictive components in the ESI+ mode

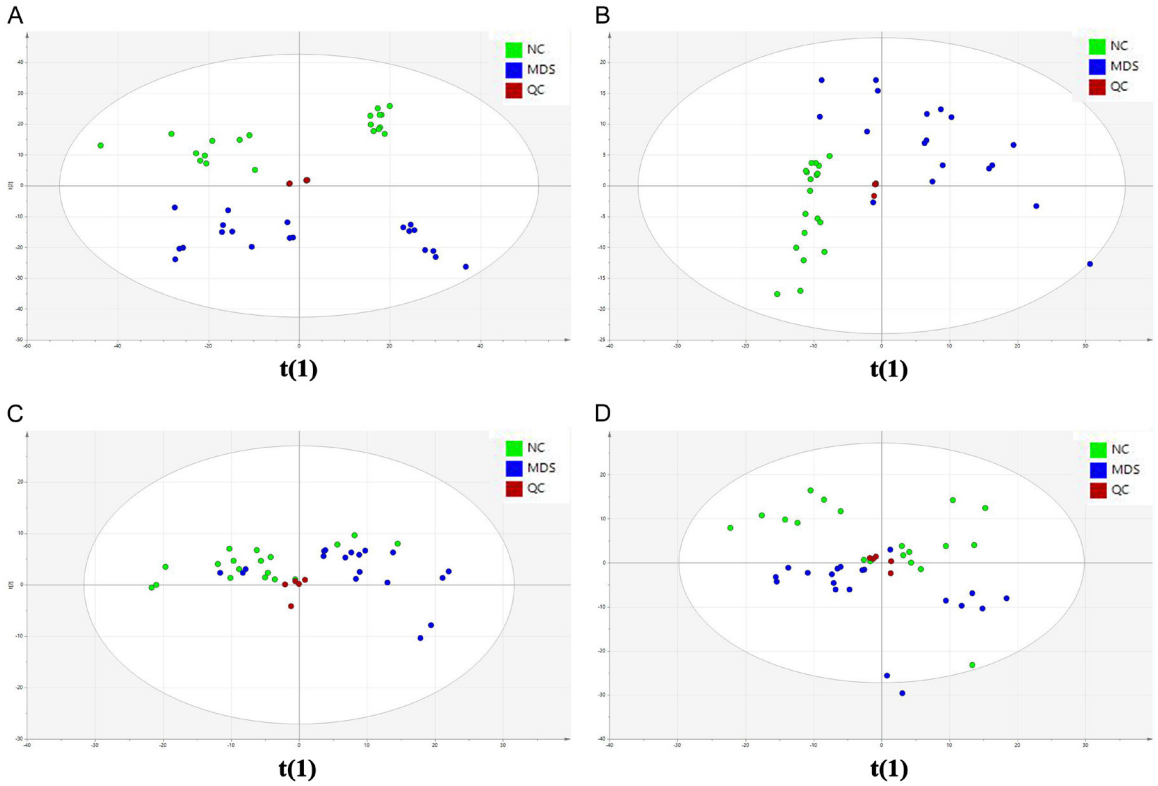

Figure 1. PCA score plots discriminating serum and urine samples between MDS and NC groups. QC samples were tightly clustered, suggesting a stable metabolic profiling platform. A. The PCA of serum under ESI+ mode. B. The PCA of serum under ESI- mode. C. The PCA of urine under ESI+ mode. D. The PCA of urine under ESI- mode. QC samples were tightly clustered, which suggested that the metabolic profiling platform was stable.

(R2Ycum = 0.966, Q2cum = 0.867) and two components in the ESI- mode (R2Ycum = 0.910, Q2cum = 0.648) in urine. We also conducted a permutation test with 100 iterations containing two predictive components to avoid overfitting [25]. The results showed that almost all the permuted Q2cum values were lower than the original values (**Figures 2B**, **2D**, **3B**, and **3D**), assuring the validity of our supervised models.

Abnormal metabolic profile in serum

In the present study, 12 metabolites exhibited significant differences in the serum between MDS subjects and healthy individuals (**Table 2**). The heat map also suggested the dynamic changes in the metabolites between the MDS and NC groups, including the expression levels for each metabolite in every sample (**Figure 4A**). The results showed that the levels of arachidonic acid, proline, pyroglutamic acid, histidinyl-aspartate, homoserine, purine, uridine, deoxyadenosine, hypoxanthine, phenylalanyl-phenylalanine, phosphatidylcholine (PC) [20:5 (5Z, 8Z, 11Z, 14Z, 17Z)/0:0], and CDP-DG (I-12:0/I-13:0) were disordered in the serum samples of the MDS group compared to healthy individuals. These metabolites are also represented as the mean and standard error of the mean on a bar graph (**Figure 5**) to evaluate the increasing or decreasing tendency of each metabolite in the MDS group.

Disordered metabolic profile in urine

Table 3 shows significant differences in the urine samples between the two groups. Compared to the metabolites in the NC group, the levels of arginine, prolyl-tyrosine, hydroxyprolyl-isoleucine, deoxyadenosine, and orotidine were decreased in the patients with MDS, while those of methionyl-alanine and adenosine were elevated. Similar trends of these metabolites were also observed on heat maps (**Figure 4B**) and bar graphs (**Figure 6**).

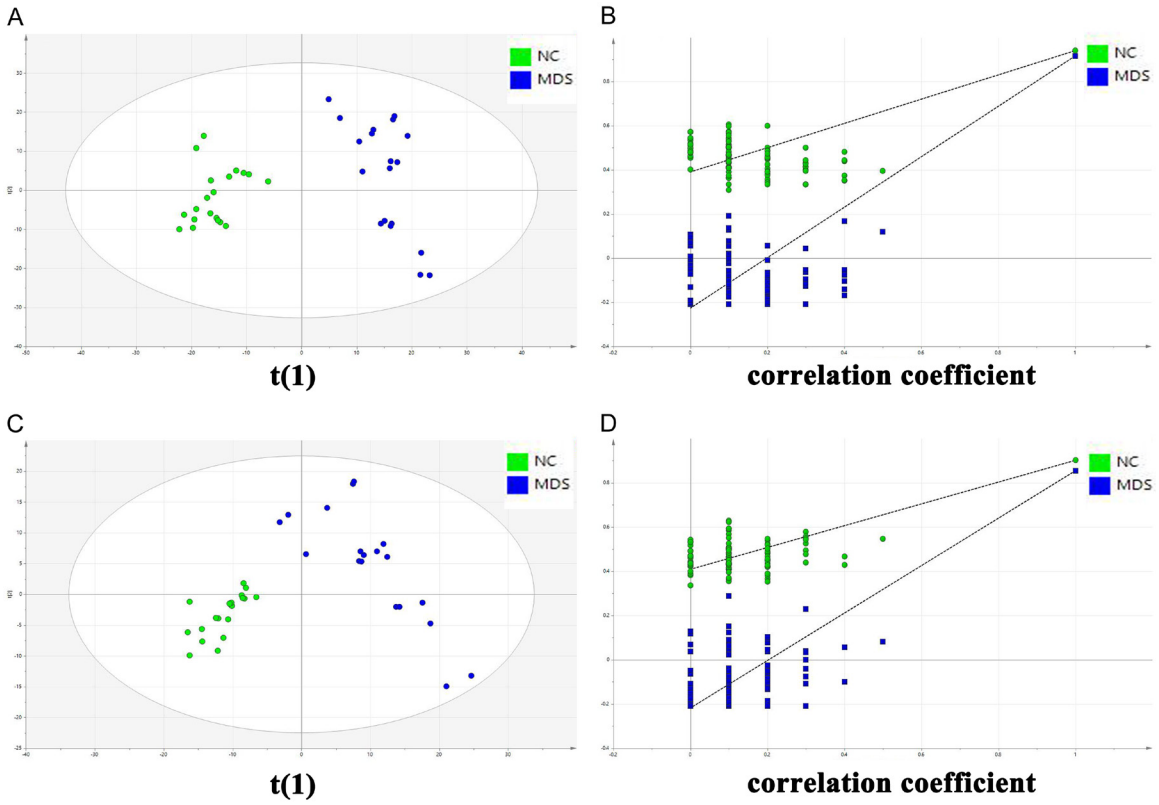

Figure 2. PLS-DA score plots and validation plots discriminating the serum samples between the MDS and NC groups. A. PLS-DA score plots discriminating serum sample of MDS and NC groups in the ESI+ mode. B. Validation plot discriminating serum sample of MDS and NC groups in the ESI+ mode. C. PLS-DA score plots discriminating serum sample of MDS and NC groups in the ESI- mode. D. Validation plot discriminating serum sample of MDS and NC groups in the ESI- mode.

Discussion

Abnormal amino acid metabolites in MDS

Amino acids are the major nutrients in the body, involved in the synthesis of proteins, fatty acids, and ketones and vital physiological processes, such as glycolysis and tricarboxylic acid circulation [26, 27]. Proline is a non-essential amino acid synthesized by glutamate, the main component of collagen, and is crucial for the normal function of bone marrow [28]. It also combines with cytoskeleton-modulating protein profilin 1 and plays a major role in regulating the metabolism of hematopoietic stem cells [29]. Importantly, abnormal proline metabolism has been reported in bone marrow-derived diseases, such as AML [30]. Pyroglutamate, a proline derivative, is the main intermediate of arginine and proline metabolism pathway together with proline [31]. Therefore, proline and pyroglutamic acid levels were abnormal in the serum of MDS patients in this study, indicating disordered arginine and proline metabolic pathways in MDS patients.

We also detected phenylalanine and aspartate-histidine abnormalities in the serum of MDS patients. Phenylalanine, together with tyrosine, synthesizes critical neurotransmitters and hormones in the body's glucose and fat metabolism [32]. Aspartate-histidine is an unstable dipeptide in the body and is rapidly degraded by enzymes into aspartic acid and histidine [33]. Aspartic acid is also closely related to ornithine cycle, which is involved in the conversion of ammonia into urea [34]. It is also involved in the synthesis of the precursors of amino acids, such as isoleucine, methionine, purines, and pyrimidine bases [35]. Histidine can form coordination compounds with iron and other metal ions to promote iron absorption, prevent anemia, initiate deamination to form oxaloacetic acid, and participate in the ATP cycle [36]. Therefore, the current results suggested that MDS patients may be compli-

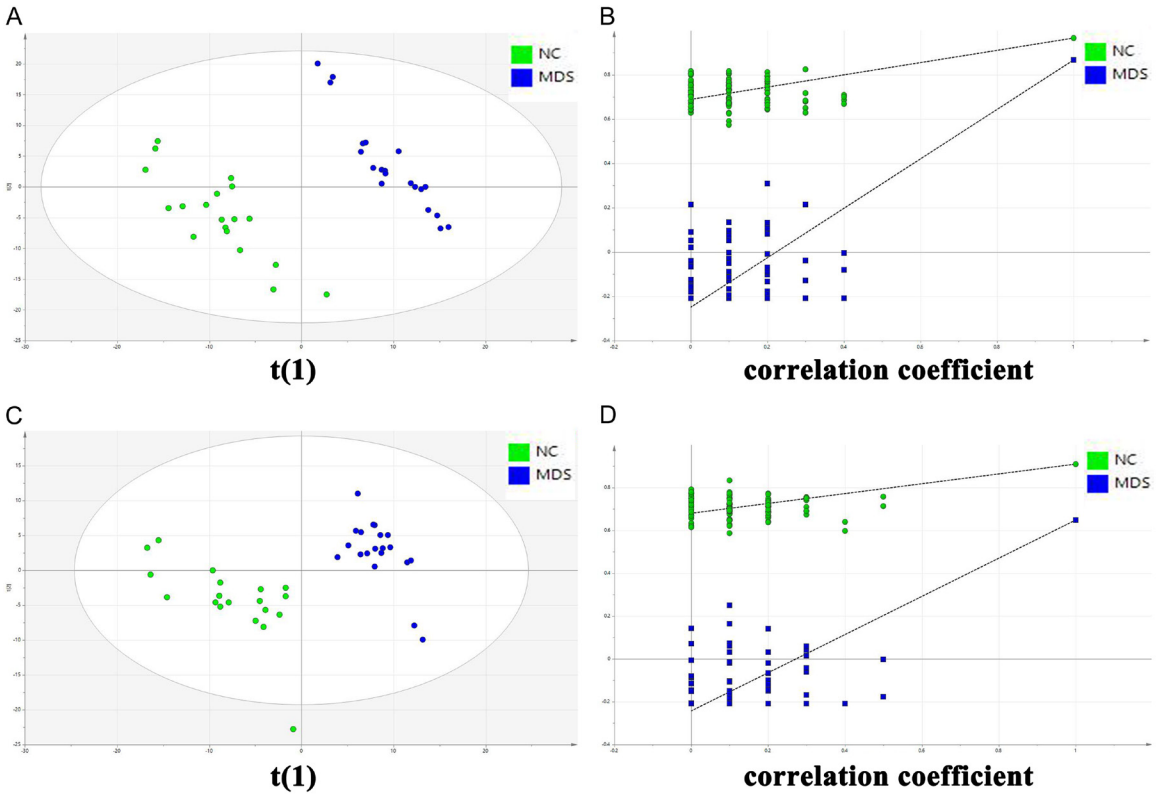

Figure 3. PLS-DA score plots and validation plots discriminating the urine samples between the MDS and NC groups. A. PLS-DA score plots discriminating urine sample of MDS and NC groups in the ESI+ mode. B. Validation plot discriminating urine sample of MDS and NC groups in the ESI+ mode. C. PLS-DA score plots discriminating urine sample of MDS and NC groups in the ESI- mode. D. Validation plot discriminating urine sample of MDS and NC groups in the ESI- mode.

Table 2. Detailed information about 12 serum abnormal metabolomics biomarkers in MDS and NC groups

Number	Name	m/z	RT(s)	P	FDR	VIP	mode
1	Proline	116.07	53.61	0.0012	0.0175	1.12	ESI+
2	Deoxyadenosine	249.15	1390.08	2.01E-07	5.10E-06	1.87	ESI+
3	Arachidonic acid	305.25	1286.58	0.0027	0.0400	1.05	ESI+
4	PC [20:5 (5Z, 8Z, 11Z, 14Z, 17Z)/0:0]	542.32	1023.87	0.0005	0.0070	1.23	ESI+
5	L-Homoserine	118.05	51.58	0.0004	0.0029	1.08	ESI-
6	Purine	119.04	51.04	0.0068	0.0479	1.19	ESI-
7	Pyroglutamic acid	128.04	60.11	2.06E-07	1.83E-06	1.38	ESI-
8	Hypoxanthine	135.03	55.11	3.74E-06	2.99E-05	1.32	ESI-
9	Uridine	243.06	56.97	8.52E-07	9.89E-06	1.02	ESI-
10	Histidinyl-aspartate	269.09	88.68	0.0080	0.0479	1.14	ESI-
11	Phenylalanyl-phenylalanine	311.14	477.01	0.0001	0.0009	1.28	ESI-
12	CDP-DG (I-12:0/I-13:0)	854.39	52.97	3.93E-05	0.0003	1.26	ESI-

cated with phenylalanine and tyrosine metabolic disorder as well as histidine and aspartic acid metabolic disorder.

The excretion of metabolites in urine indirectly reflects the metabolic processes in the body. In the present study, we found abnormal levels of arginine, proline-tyrosine, hydroxyproline-isoleucine, and methionine-alanine in the urine of MDS patients. Arginine, an essential amino acid, is involved in ornithine cycle and is the substrate for the synthesis of nitric oxide. Cull

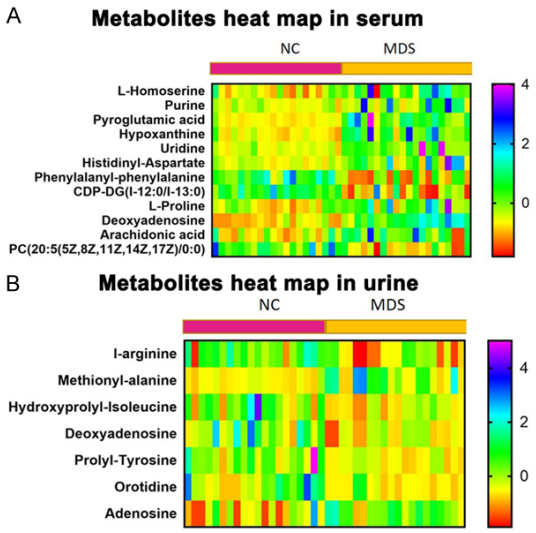

Figure 4. Heat map demonstrating the dynamic changes in the biomarkers of the MDS and NC groups in the serum and urine, which illustrates expression levels of each metabolite in every sample. Each box represents a sample. A. Heat map demonstrating dynamic changes in biomarkers for MDS and NC groups in serum, which illustrates expression levels of each metabolite in every sample. Each box represents a sample; B. Heat map demonstrating dynamic changes in biomarkers for MDS and NC groups in urine.

et al. [37] reported that abnormal arginine metabolism occurred in the bone marrow cells of MDS patients, which was associated with the immune dysregulation of MDS patients. Hydroxyproline is an amino acid derivative with a structure and function similar to that of proline [38]. Methionine is activated by ATP and is continuously converted to S-adenosylmethionine, which is then demethylated to S-adenosylhomocysteine [39]. Alanine is one of the major gluconeotropic substrates in the human body. It produces glucose and converts amino nitrogen into urea [40]. Therefore, these abnormal amino acid metabolites in the urine of MDS patients also confirmed the above conclusion. These metabolic pathway disorders may be related to anemia, energy metabolism abnormality, immune disorders, and leukemia transformation in patients with MDS.

Disordered nucleotide metabolites in MDS

Nucleotides are the final products of purine and pyrimidine metabolism. Some nucleotides (such as hypoxanthine) depend mainly on the metabolic processes in the body, while others (such as uridine) depend on the nutritional factors [41]. Purines consist of adenine, guanine, hypoxanthine, and xanthine. Adenosine is first converted to deoxyadenosine and then converted to hypoxanthine, which is then converted to adenine or guanineusing hypoxanthine-guanine phosphate ribose transferase (HPRT) or adenine phosphate ribose transferase [42, 43]. These are the scaffold substrates of nucleic acid, coenzyme, allosteric regulator, and cell energy intermediates that play a major role in energy supply, metabolism regulation, and composition of coenzymes [43]. The current results showed that the purines, deoxyadenosine, and hypoxanthine, are increased in serum samples, suggesting purine metabolism disorder in MDS patients. Strikingly, increased adenosine and decreased deoxyadenosine in urine samples also indirectly supported this conclusion.

Pyrimidine is the precursor of nucleic acids with crucial physiological roles in the cell. Pyrimidines include uracil, cytosine, and thymine. Uracil plus a ribose forms uridine [44], which is an essential pyrimidine nucleotide for RNAs synthesis; it maintains cellular function and energy metabolism [45]. Orotidine is an intermediate in uridine synthesis and is converted to uridine monophosphate (UMP) by orotidine monophosphate (OMP) decarboxylase catalysis [46]. The uridine biosynthetic pathway disorder leads to orotic aciduria. Uridine also acts as a substrate to provide CDP-DG for PC biosynthesis through the Kennedy pathway [47]. PC is not only the main component of the membrane lipid bilayer structure but also the storage lipid of arachidonic acid [48]. Arachidonic acid is a direct precursor of prostaglandin and thrombin, which helps to regulate the WBC function and PLT activation [49]. Homoserine is a critical metabolic substance that participates in the biosynthesis of purines and pyrimidines [50]. In the present study, uridine, homoserine, CDP-DG (I-12:0/I-13:0), and PC [20:5 (5Z, 8Z, 11Z, 14Z, 17Z)/0:0] were abnormal in the serum of MDS patients, while orotidine was abnormal in the urine samples. These findings suggested that hematopoietic system disorders and malignant transformation of leukemia in MDS patients might be related to the abnormal metabolism of nucleotides and disordered Kennedy's metabolic pathway.

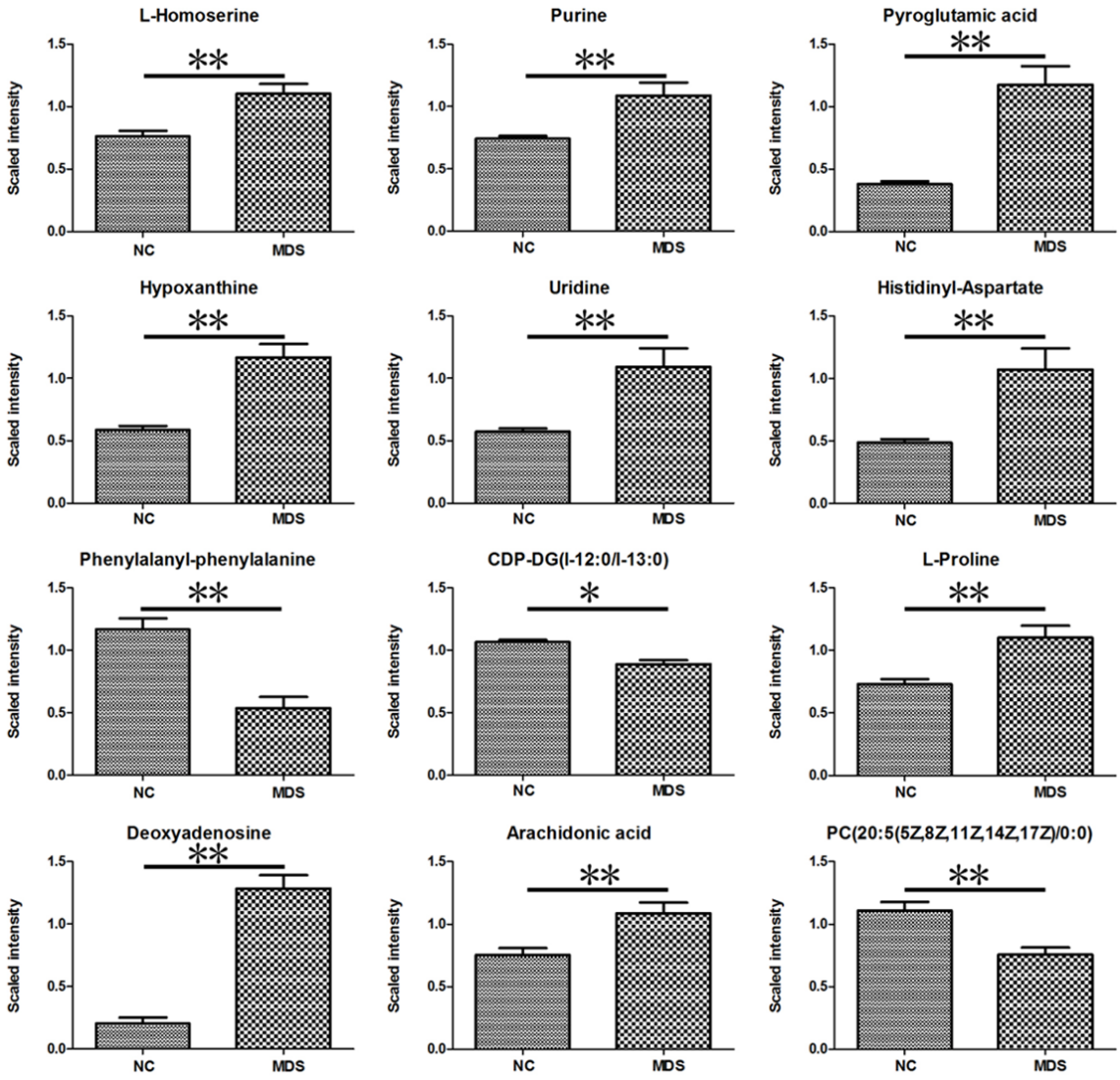

Figure 5. Metabolite profiles of potential biomarkers in the serum of MDS and NC groups (*P<0.05, **P<0.01).

Table 3. Detailed information about 7 urine abnormal metabolomics biomarkers between MDS and NC groups

Number	Name	m/z	RT(s)	P	FDR	VIP	mode
1	l-arginine	189.14	52.53	0.0007	0.0126	1.6989	ESI+
2	Methionyl-alanine	221.10	57.80	0.0053	0.0344	1.6495	ESI+
3	Hydroxyprolyl-Isoleucine	245.15	59.41	0.0024	0.0203	1.4209	ESI+
4	Deoxyadenosine	252.11	60.19	0.0035	0.0344	1.5765	ESI+
5	Prolyl-tyrosine	279.13	51.93	0.0002	0.0106	1.4664	ESI+
6	Orotidine	289.07	887.47	0.0062	0.0350	1.3691	ESI+
7	Adenosine	266.09	55.165	0.0006	0.0094	1.7372	ESI-

Nevertheless, the present study has some limitations. Firstly, the small number of cases included in this study might bias the results. Secondly, the inability to control the type of food consumed by patients prior to sample collection might also render bias to the results.

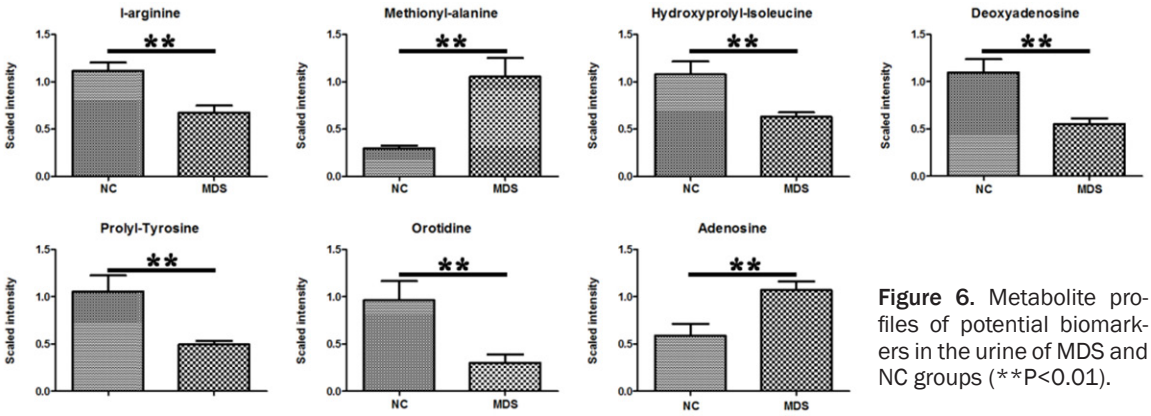

Figure 6. Metabolite profiles of potential biomarkers in the urine of MDS and NC groups (**P<0.01).

Finally, we did not identify the abnormal metabolites, which will be the focus of our future studies.

Conclusion

In the current study, abnormal metabolites were investigated using LC-QTOF-MS technique in the blood and urine of MDS patients. Consequently, 19 potential biomarkers related to MDS were identified in the ion ESI+/- modes, which belonged to the amino acid and nucleotide metabolic pathways, respectively. The identification of the potential biomarkers and the related metabolic pathways provided a valuable basis for the study of the pathogenesis of MDS, thereby offering the possibility of early diagnosis and treatment of the diseases in the future.

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Disclosure of conflict of interest

None.

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