Plasma amino acid profiling of cancer patients with abnormal Savda based on high-performance liquid chromatography

Batur Mamtimin, Kedena, Bin Kong, Ayshamgul Hasim, Aynur Matsidik, Mawlanjan Hizbulla, Nazuk Kurbantay, Halmurat Upur

**Abstract**

**OBJECTIVE:** To investigate metabolic signatures in plasma of cancer patients with abnormal Savda using plasma-free amino acid profiles, and to evaluate the diagnostic potential of these profiles for the detection and explanation of the mechanisms of different symptoms in traditional Uyghur medicine.

**METHODS:** Plasma samples from cancer patients with abnormal Savda (n=85) or non-abnormal Savda (n=105) and a healthy control group (n=65) were analyzed using high-performance liquid chromatography (HPLC). Orthogonal projection to latent structures with discriminant analysis was used for the classification and prediction of abnormal Savda, and spectral profiles were subjected to Student's t-tests to assess statistical significance.

**RESULTS:** Compared with the healthy group, the levels of aspartic acid, glutamate, glycine, histidine, arginine, threonine, alanine, proline, methionine, isoleucine, leucine and phenylalanine decreased significantly in plasma of cancer patients with abnormal Savda (all \( P < 0.05 \)). Serine, cystine, tyrosine, valine and lysine levels showed no significant differences (all \( P > 0.05 \)). Compared with non-abnormal Savda syndrome patients, abnormal Savda syndrome patients showed high concentrations of glutamate, serine, valine, isoleucine, leucine and phenylalanine (all \( P < 0.05 \)). The remaining plasma amino acids showed no significant differences (all \( P > 0.05 \)).

**CONCLUSION:** Plasma-free amino acid profiling has the potential to assist in understanding and determining abnormal Savda. A HPLC-based metabonomic platform could be a powerful tool for the classification of symptoms in traditional medicine.

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**Key words:** Amino acids; Medicine, Uyghur traditional; Chromatography, High pressure liquid; Abnormal Savda

**INTRODUCTION**

Disease treatment in traditional Uyghur medicine is characterized by abnormal body fluid types. According to the body fluid theory, abnormal Savda is a special state and symptom. As a pathological product, it...
can increase the incidence of complex and intractable diseases like cancer.\textsuperscript{14} A vast quantity of clinical data suggests that 72\% of cancer patients with abnormal Savda have other diseases including diabetes and hypertension, and the number of asthma patients with abnormal Savda is far higher than the number of patients with the three other abnormal body fluids, these being abnormal Belgheim, abnormal Sapra and abnormal Kan.\textsuperscript{7} This perhaps suggests that abnormal Savda is closely related to complex disease occurrences and development.

Cancer is a typically complex disease. Early and ultra-early detection is suggested for improving the survival rate of cancer patients. Finding metabolic markers based on metabonomics for cancer and other complex diseases and early classification diagnosis and treatment will be important in future research.

Metabonomics is a new method for studying dynamic changes in endogenous metabolites in biosystems. This process applies chemical analysis techniques combined with chemometric methods using various advanced techniques such as nuclear magnetic resonance (NMR), high-performance liquid chromatography (HPLC)/mass spectrometry to detect and measure low molecular weight metabolites in human body fluids and tissues.\textsuperscript{7,8} We hypothesized that the occurrence and development processes of abnormal Savda result from a metabolic abnormality, and that metabonomics will help provide information on the concept of different body fluid syndromes.

In our previous study, we used metabonomic analysis to examine plasma samples from cancer patients with abnormal Savda and non-abnormal Savda by NMR. The results indicated that there were differences in some kinds of metabolites in patients with different syndromes, including amino acids.\textsuperscript{3-6} However, the degree of variation of the amino acid content was unclear. Protein synthesis and normal cell growth require amino acids. Understanding whole-body amino acid metabolism may help explain the biological basis of abnormal Savda.

In this study, we used HPLC to determine plasma-free amino acid profiles of healthy participants, patients with abnormal Savda and patients with non-abnormal Savda. We also evaluated the possibility of using this information for the detection of, and to explain the mechanisms for, the different symptoms treated with traditional Uyghur medicine.

**MATERIALS AND METHODS**

**Chemicals and reagents**

Amino acid standards were purchased from Waters (Milford, MA, USA). These were: aspartic acid (Asp), glutamate (Glu), serine (Ser), glycine (Gly), histidine (His), arginine (Arg), threonine (Thr), alanine (Ala), proline (Pro), cysteine (Cys), tyrosine (Tyr), methionine (Met), valine (Val), isoleucine (Ile), leucine (Leu), lysine (Lys) and phenylalanine (Phe). An AccQ-Fluor reagent kit was also obtained from Waters. Acetonitrile and methanol (HPLC grade) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Sulfuric acid, methanol and n-hexane were analytical grade and were purchased from Tianjin Chemical Reagent Factory (Tianjin, China). The water used in the study was ultra-pure and was produced using a Milli-Q system (Millipore Corp., Boston, MA, USA).

**Sample collection and preparation**

Sixty-five healthy volunteers who were confirmed by medical examination and 190 cancer patients from the Fourth Affiliated Hospital of Xinjiang Medical University using the staging system of the American Joint Committee on Cancer were enrolled in the study. Exclusion criteria included individuals with cardiovascular diseases, hepatic diseases, renal diseases or inflammatory diseases. Pregnant women were also excluded. Cancer diagnosis was established in patients immediately prior to the commencement of the study and therefore no treatment had yet been administered. Diagnosis of abnormal Savda syndrome and non-abnormal Savda syndrome was made based on physical examination, according to the symptoms and signs described in traditional Uyghur medicine,\textsuperscript{2,4} and confirmed by three experienced physicians.

In Uyghur medicine, the main clinical manifestations of abnormal Savda are a fine and slow pulse, blue and deep orbit, bleak face, bitter taste, gray or black tongue fur, rough skin, low body temperature, less frequency of urination (but more volume), insomnia and dreams and nightmares. The other cancer patients with non-abnormal Savda syndrome were diagnosed according to what is written in traditional Uyghur medicine. There were 85 cases in the abnormal Savda syndrome group and 105 cases in the non-abnormal Savda syndrome group. The average age of participants was (50±10) years.

Blood samples were collected from participants before breakfast. Plasma was obtained by centrifugation of blood samples at 3500 \( \times g \) for 10 min, and was immediately stored at -80°C until used for HPLC.

Samples were allowed to thaw prior to analysis. A standard sample was prepared by mixing 200 \( \mu L \) plasma with 400 \( \mu L \) acetonitrile. The mixture was shaken vigorously for 30 s and then centrifuged at 12000 \( \times g \) for 15 min at 4°C. The supernatant was dried under nitrogen at 40°C. Water was then added (200 \( \mu L \)) and the sample shaken vigorously for 30 s and filtered using a micropore filter membrane. A 10-\( \mu L \) filtration sample was added to 70 \( \mu L \) of borate buffer and 15 \( \mu L \) AQC reagent and placed in a 55°C water bath for 10 min before chromatographic analysis.

The study protocol was approved by the Ethics Committee of Xinjiang Medical University. All participants gave written informed consent to participate in the study.

**Apparatus and operation conditions**

A Waters c2695 series HPLC system (Milford, MA, USA) with a 2475 fluorescence detector was used for
analysis. Chromatographic separation was performed on an Inertsil ODS-SP (C18, 5 μm particle size, 4.6 mm × 250 mm; Shimadzu, Kyoto, Japan) maintained at 35 °C with a flow rate of 1.0 mL/min. The excitation wavelength was 235 nm and the emission wavelength was 395 nm. Mobile phase A consisted of methanol-acetonitrile-water at 1:3:1 (v/v), and B was acetate buffer salt. Using a gradient elution of 100% B at 0-5 min; 98% B at 5-6 min; 94% B at 6-15 min; 91% B at 15-19 min; 79% B at 19-32 min; 55% B at 32-34 min; 45% B at 34-38 min; 0% B at 38-45 min. The injection volume was 5 μL.

Data analysis

After the chromatographic peak area was normalized, pattern recognition analysis was carried out on data sets using SIMCA-P+ software (Version 11.0, Umetrics Inc., Umea, Sweden). A supervised multivariate data analysis tool called the orthogonal projection to latent structures with discriminant analysis (OPLS-DA) was used, with unit-variance scaling to classify the abnormal Savda syndrome cancer group, the non-abnormal Savda syndrome cancer group and the healthy group. This was constructed using amino acid concentrations in plasma as the X matrix and class information identifier for the different groups as the Y variables. In the current study, R² and Q² were used to describe the quality of the OPLS-DA model. Total explained variation for the X matrix is R², and predictability of the model is Q². All amino acid concentrations are expressed as mean ± standard deviation (x̄ ± s). Student’s t-tests was performed by SPSS (Version 16.0, Chicago, IL, USA) software to analyze alterations in amino acid levels among the different groups. A P-value <0.05 was considered statistically significant.

RESULTS

Typical HPLC chromatography

Results from HPLC chromatography showed that 17 amino acids were separated effectively in 30 min, the peaks of interest were well separated, and there was no interference from other amino acids and endogenous compounds in the plasma (Figure 1).

**OPLS-DA analysis**

R²X and Q² were 0.43 and 0.42, respectively. The value of Q² showed encouraging prediction ability for this OPLS-DA model (Figure 2). The results indicated that the OPLS-DA model had good capability for the classification of the different symptoms of cancer patients. Plasma-free amino acid profiling has great potential to improve the screening and diagnosis of different symptoms of cancer and to help with understanding abnormal Savda in traditional Uyghur medicine.

**Plasma-free amino acid profiles**

Compared with the healthy group, the levels of Asp, Glu, Gly, His, Arg, Thr, Ala, Pro, Met, Ile, Leu and Phe decreased significantly in plasma of cancer patients with abnormal Savda (all P<0.05), but Ser, Cys, Tyr, Val and Lys levels showed no significant differences (all P>0.05). Compared with non-abnormal Savda syndrome patients, abnormal Savda syndrome patients had high concentration of Glu, Ser, Val, Ile, Leu and Phe (all P<0.05) but the other plasma amino acids showed no significant differences (all P>0.05; Table 1).

**DISCUSSION**

The body fluid (Hilit) theory of traditional Uyghur medicine considers that body fluid produced in the liver by various nutrients provides energy for the whole organism. According to this theory, the body fluid includes Savda, Belghem, Sapra and Kan, and abnormal changes in body fluids are the basis of diseases, which is called abnormal Hilit. Based on the symptoms, abnormal Hilit is divided into abnormal Savda, abnormal Belghem, abnormal Sapra and abnormal Kan. Abnormal Savda is a special syndrome and is considered as

![Figure 1 Typical HPLC chromatography profiles of plasma-free amino acids](image-url)
one of the origins of many complex diseases such as cancer, diabetes, hypertension and asthma. Amino acids are an important class of substances in the activities of life with a wide range of biological functions: the composition of proteins, involved in energy metabolism and precursors of nitrogen-containing substances.

Table 1: Plasma-free amino acid profiles of cancer patients with abnormal Savda or non-abnormal Savda and healthy participants (mmol/L, \( \bar{x} \pm s \))

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Healthy group</th>
<th>Abnormal Savda</th>
<th>Non-abnormal Savda</th>
<th>( P ) value</th>
<th>( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asp</td>
<td>0.037±0.033</td>
<td>0.006±0.006</td>
<td>0.006±0.004</td>
<td>0.000</td>
<td>0.329</td>
</tr>
<tr>
<td>Glu</td>
<td>0.310±0.279</td>
<td>0.075±0.048</td>
<td>0.061±0.037</td>
<td>0.000</td>
<td>0.009</td>
</tr>
<tr>
<td>Ser</td>
<td>0.238±0.497</td>
<td>0.162±0.162</td>
<td>0.120±0.136</td>
<td>0.092</td>
<td>0.025</td>
</tr>
<tr>
<td>Gly</td>
<td>0.257±0.165</td>
<td>0.170±0.118</td>
<td>0.150±0.099</td>
<td>0.000</td>
<td>0.100</td>
</tr>
<tr>
<td>His</td>
<td>0.119±0.089</td>
<td>0.074±0.036</td>
<td>0.082±0.097</td>
<td>0.000</td>
<td>0.234</td>
</tr>
<tr>
<td>Arg</td>
<td>0.085±0.045</td>
<td>0.066±0.038</td>
<td>0.074±0.040</td>
<td>0.001</td>
<td>0.068</td>
</tr>
<tr>
<td>Thr</td>
<td>0.183±0.186</td>
<td>0.088±0.087</td>
<td>0.074±0.086</td>
<td>0.000</td>
<td>0.189</td>
</tr>
<tr>
<td>Ala</td>
<td>0.797±0.961</td>
<td>0.237±0.250</td>
<td>0.240±0.292</td>
<td>0.000</td>
<td>0.464</td>
</tr>
<tr>
<td>Pro</td>
<td>0.085±0.063</td>
<td>0.053±0.053</td>
<td>0.057±0.040</td>
<td>0.000</td>
<td>0.384</td>
</tr>
<tr>
<td>Cys</td>
<td>0.029±0.018</td>
<td>0.027±0.056</td>
<td>0.018±0.041</td>
<td>0.449</td>
<td>0.113</td>
</tr>
<tr>
<td>Tyr</td>
<td>0.059±0.051</td>
<td>0.077±0.098</td>
<td>0.068±0.087</td>
<td>0.095</td>
<td>0.311</td>
</tr>
<tr>
<td>Val</td>
<td>0.207±0.142</td>
<td>0.225±0.217</td>
<td>0.173±0.092</td>
<td>0.287</td>
<td>0.024</td>
</tr>
<tr>
<td>Met</td>
<td>0.110±0.183</td>
<td>0.025±0.012</td>
<td>0.028±0.033</td>
<td>0.000</td>
<td>0.243</td>
</tr>
<tr>
<td>Iso</td>
<td>0.232±0.168</td>
<td>0.130±0.082</td>
<td>0.103±0.066</td>
<td>0.000</td>
<td>0.007</td>
</tr>
<tr>
<td>Leu</td>
<td>0.180±0.155</td>
<td>0.131±0.085</td>
<td>0.104±0.067</td>
<td>0.007</td>
<td>0.008</td>
</tr>
<tr>
<td>Lys</td>
<td>0.076±0.052</td>
<td>0.077±0.071</td>
<td>0.070±0.058</td>
<td>0.458</td>
<td>0.172</td>
</tr>
<tr>
<td>Phe</td>
<td>0.107±0.066</td>
<td>0.068±0.036</td>
<td>0.059±0.029</td>
<td>0.000</td>
<td>0.015</td>
</tr>
</tbody>
</table>

Notes: \( P \): cancer patients with abnormal Savda compared with healthy participants; \( P \): cancer patients with abnormal Savda compared with cancer patients with non-abnormal Savda. Asp: aspartic acid; Glu: glutamate; Ser: serine; Gly: glycine; His: histidine; Arg: arginine; Thr: threonine; Ala: alanine; Pro: proline; Cys: cystine; Tyr: tyrosine; Met: methionine; Val: valine; Ile: isoleucine; Leu: leucine; Lys: lysine; Phe: phenylalanine.
compounds such as neurotransmitters, nucleic acids and coenzymes. A variety of pathological conditions can lead to changes in amino acid metabolism. Therefore, studying the metabolism of amino acids in the body under physiological and pathological conditions contributes to the understanding of the pathological mechanisms of various diseases, as well as diagnosis and treatment.

Metabolism in cancer cells is significantly altered compared with normal cells, and these changes are also reflected in the plasma amino acid profiles. It has been reported that plasma amino acid profiles can be used to discriminate between patients with cancer and healthy controls. Detecting amino acid profiles in plasma could potentially be useful for the diagnosis of different cancers.

To better understand amino acid metabolism differences in cancer patients with abnormal Savda, we analyzed plasma-free amino acid concentrations using HPLC. We performed plasma amino acid profiling to identify marker metabolites and to assess whether these marker metabolites could be used for abnormal Savda detection in patients with complex diseases. The results showed that the model used identified patients with abnormal Savda, suggesting that plasma amino acid profiling may be useful for the diagnosis of different syndromes in traditional Uyghur medicine.

Savda is produced in the liver by various nutrients according to Uyghur medicine. Amino acids are a necessary nutritional resource, but variations in endogenous concentrations correlate with disease. Therefore, plasma amino acids are the most suitable candidates for identifying abnormal Savda as they play essential physiological roles both as basic metabolites and metabolic regulators.

Tumor tissue continues to uptake amino acids for protein synthesis and cell proliferation, and plasma-free amino acids are the major uptake source. Metabolism of specific amino acids is known to be associated with specific organs, such as muscles, the liver or kidneys. Changes in amino acid levels are affected by their metabolism in, and excretion from, multiple organs of the body, and amino acids are frequently identified compounds among whole metabolites in the blood in relation to abnormal Savda.

With the rapid growth of a tumor, the tumor and the body compete for nutrients, especially amino acids, which inevitably lead to amino acid metabolism disorders. In this study, we found that the amino acid metabolism of patients with abnormal Savda was significantly different not only to the metabolism of healthy volunteers but also with non-abnormal Savda patients; a finding which lends support to the body fluid theory.

It has been reported that patients with several different types of cancers have low levels of plasma branched-chain amino acids (BCAA), including Val, Leu, and Ile. The results of the current study are consistent with these reports. It is also of interest that there were different BCAA concentrations observed in plasma of patients with different symptoms.

In this study, cancer patients with abnormal Savda had higher plasma BCAA levels than non-abnormal Savda patients. Liver and muscle are important tissues for amino acid metabolism, so these results may be suggestive of increased protein degradation in skeletal muscle of abnormal Savda patients being higher than that of non-abnormal Savda patients. BCAA are central in the maintenance of skeletal muscle protein metabolism. Traditional Uyghur medicine considered that patients with abnormal Savda suffer weight loss and energy metabolism disorders. It is therefore important to examine BCAA metabolism in the abnormal Savda state, where muscle wasting is prominent. Skeletal muscle wasting indicates that overall protein catabolism is greater than anabolism in cancer patients. Our previous study indicated that patients with abnormal Savda showed greater muscle wasting than non-abnormal Savda patients. BCAA are also thought to affect glucose metabolism. Cancer patients experiencing weight loss showed increased rates of gluconeogenesis. It is therefore possible that patients with abnormal Savda might have higher gluconeogenesis rates than non-abnormal Savda patients.

BCAA are not only a constituent of protein, but also a source of Glu. It was observed in the current study that abnormal Savda patients had higher concentrations of Glu in plasma than non-abnormal Savda patients. Glu is an amino acid similar to glutamine. In the blood, Glu is present at a level approximately 25% of that of glutamine. Glutamine is a key substrate in many synthetic and metabolic processes, such as the breakdown of skeletal muscle protein, and is also a source of alpha-ketoglutarate, which plays an anaplerotic role in the tricarboxylic acid cycle as citrate and malate. Ser is a non-essential amino acid. It plays a role in the metabolism of fats and fatty acids and muscle growth, and also in the synthesis of the cell membrane, muscle tissue and the sheath of nerve cells. It is possible that plasma Ser concentrations of patients with abnormal Savda may be related somehow with the degradation of muscle tissue.

According to the body fluid theory, body fluids are derived from the liver. The liver is the main organ involved in protein and amino acid metabolism, and affects protein metabolism by the formation of plasma proteins, amino acid interconversion, deamination of amino acids, and urea synthesis. Amino acid abnormalities are common even in patients who have liver cirrhosis. For that reason, amino acid metabolic abnormalities in the liver become more severe with abnormal Savda. Phe is important for assessing liver metabolism and the severity of liver dysfunction. High Phe concentrations in plasma of abnormal Savda patients may be related with liver dysfunction.

Plasma is an essential body fluid which contains all kinds of information about the pathological changes of
organs and tissues, including information about the mechanisms of different symptoms like abnormal Savda. Therefore, correlation-based network analysis of plasma amino acids may help to uncover specific pathological states and aid in establishing strategies for the early detection of abnormal Savda.

In this study, we examined amino acid metabolism of cancer patients with different traditional Uyghur medicine symptoms using HPLC analysis. We found that the amino acid balance in cancer patients with abnormal Savda was significantly different not only to that of healthy individuals but also cancer patients with non-abnormal Savda. The results suggest that amino acid profiling may detect latent variances and may be able to define chemometric models for the prediction of abnormal Savda. The HPLC technique described in this study may have great potential to assist in the understanding of the substance basis of traditional medicine theory.

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REFERENCES