Metabolomics studies of chronic atrophic gastritis cold and heat syndrome

M.L. Wang[1,*], C.L. Zheng[2,*], Y.W. Zhou[2,#], T.G. Liu[3], Y.B. Li[3], H. Xie[3], Y. Chang[4], W.C. Liu[3], G.G. Chen[3], X.L. Sun[3], Y.H. Zhao[5,*], Z.H. Li[6,*]

[1] *Beijing Jiangong Hospital, Beijing 100054,China*
[2] *Beijing Gulou Hospital of Traditional Chinese Medicine, Beijing 100009, China*
[3] *Beijing University of Chinese Medicine, Beijing 100029,China*
[4] *Beijing Research Center of Urban System Engineering, Beijing 100037,China*
[5] *School of Traditional Chinese Medicine, Capital Medical University, Beijing 100069, China*
[6] *Dongzhimen Hospital, Beijing University of Chinese Medicine, Beijing 100007, China*

The objective of this work is to study the specific plasma metabolites of chronic atrophic gastritis cold and heat syndrome, an d search the relevance pattern with cold-heat syndrome. Collect general data and blood samples of chronic atrophic gastritis cold- heat syndrome patients through clinical cross-sectional investigation, and resort GC-MS analytic technique to analyze the patient's metabolites and change of corresponding metabolites spectrum group. Then use many kinds of data-mining methods to discuss the corresponding relation between cold-heat syndrome and specific metabolites. The true positive rate of this decision tree modeling is 81.5 %, which is established by compounds whose retention time are 33.16, 21.83, 33.26, 34.69. chronic atrophic gastritis cold and heat syndrome will produce specific metabolites that may provide an objective basis for judging cold-heat syndrome. Accordingly, new ideas and methods are provided for pattern identification of traditional Chinese medicine (TCM) and deep research on symptoms.

Keywords*:* chronic atrophic gastritis, cold syndrome, heat syndrome, metabolomics, data mining 1

INTRODUCTION

Chronic atrophic gastritis is a chronic disease with atrophy of the stomach mucosa, leading to loss of gastric glandular cells, incrassation of mucosa muscle layer and their eventual replacement by intestinal and fibrous tissues. CAG is listed as the stomach precancerous disease or precancerous condition, and on its basis concomitant with intestinal metaplasia (IM) and hyperplasia (Dys) that regarded as gastric cancer and precancerous lesion of gastric cancer (PLGC). In China, as working pressure increases and life pace becomes faster, the incidence of chronic atrophic gastritis increased year by year, which has a serious impact on people's life quality. Traditional Chinese medicine has accumulated rich experience in the thousands of years of clinical practice. Traditional Chinese Medicine provided a complementary and alternative therapeutic regimen to treating CAG. It uses a holistic concept to balance whole body, being different to western medicine whose treatment of chronic atrophic gastritis.

TCM has a history of more than 2000 years to fight with CAG. Chronic atrophic gastritis, according to its symptoms and signs, can be assigned to "gastric stuffiness", "stomach duct pain". Its syndrome differentiation can be basically divided into stomach cold and stomach heat. Syndrome differentiation is premise of treatment (diagnosis, prescription and drugs) whose accuracy is the key to clinical effect. In recent decades, people kept looking for the objective index of syndrome in order to reveal its essence, from multilevel aspect such as physiological, biochemical, immunity, cell pathology, ultrastructure, genomics and protrinology. However, the research results indicate that characteristic of the indicators is not manifest that it is difficult to find the specific indicators, while many of the chosen indicators are difficult to promote in clinical applications [1].

It is obvious that the specific indicator of "syndrome" is more likely the changes of a group of indicators, not just one or two "golden indicators". But by using the traditional method cannot find a group of specific indicators. And previous studies only associate the results with syndrome, lacking correlation analysis between the changes of characteristic indicators and classical metabolic pathway, so that making it difficult to clarify the nature of syndrome and its evolutionary mechanism. Therefore, the exploration of syndrome related metabolites group is undoubtedly significant to solve the key problem of syndrome biological basis.

With life science having entered post genomics era in nowadays, metabonomics technology not only overcomes genomics, proteomics and other technical limitation, but also possesses enormous

---

advantage on holistically discerning the whole life process and dynamic change through the metabolic contour analysis [2,3,4], which fit close the holism traditional Chinese medicine emphasizes. Thereby, it provides new thinking and methods for syndrome research.

At present, cold syndrome and heat syndrome of chronic atrophic gastritis has made some progress on biology basic research. For instance, the researchers found the stomach heat syndrome patients, compared with stomach cold syndrome patients, PGE2 and 6-keto-PGF1αcontent in their gastric juice has a more obvious increase. Gastric heat syndrome patients appear more obvious congestion edema because of gastric mucosa vessels hemangiectasis and blood flow increase. In addition, scholars have found in the gastric juices of the stomach cold and heat syndrome, the Na+ concentration change is closely related to gastric mucosal barrier [5,6].

Li Shao and some people studied the "cold syndrome" and "heat syndrome" on molecular basis in neuroendocrine immune system, thought that "cold syndrome" may relate to hormone while "heat syndrome" may possibly relate to immune factor. The two relate each other by neurotransmitter, preliminary reveals the molecular pathways of cold and heat syndrome in rheumatoid arthritis, so that confirms the objective existence of cold and heat syndrome [7].

Therefore, this research will take chronic atrophic gastritis cold and heat syndrome as object, give the patients' organizations metabolite spectral analysis to get "syndrome-related metabolic spectrum group", combine data-mining method that is suitable for TCM syndrome data characteristics to find out specific signature metabolites, use bioinformatics methods to analyze the function of the biomarkers to preliminary reveal microscopic metabolites and disease and syndrome association laws, and reflect CAG cold and heat syndrome connotation by stomach tissue signature metabolic product in order to achieve objectification of syndrome differentiation and treatment.

MATERIALS AND METHODS

Standard of Diagnosis

CAG cold and heat syndrome disease and syndrome diagnostic standard basis
1) CAG Diagnosis Standard
New classification of chronic gastritis - Sydney system and revised Sydney system (1997)
*Endoscopic classification of CAG (national gastritis forum amendment, 1983)
2) Syndrome Diagnostic Standard

CAG syndrome differentiation standard refers to deficiency syndrome differentiation of TCM reference standard in 1986 and TCM diagnostics and relevant reports [8,9,10].

Stomach cold syndrome: main syndrome (l) stomach duct pain with heat preference, the pain will release when get heat; (2) watery stool; (3) stomach duct distention and fullness, obvious abdominal distension after noon; (4) pale or blue tongue body, white and moist tongue fur. Minor syndrome (l) exhausted; (2) sunken and fine pulse or slow pulse; (3) chills and cold limbs. People with three main syndromes and more than one minor syndrome belong to stomach cold syndrome.

Stomach heat syndrome: main syndrome (1) red tongue, yellow or less tongue fur; (2) pyrosis or stomach duct distention and fullness; (3) dry stool; (4), dry mouth, bitter taste in the mouth; (5) swift digestion with rapid hungering. Minor syndrome (1) noisy stomach or acid regurgitation; (2) slippery and string like pulse or rapid pulse. People with two main syndromes (the first one is requisite) and more than one minor syndrome belong to stomach heat syndrome.

Inclusion Criteria (satisfy all the following items)

- accord with CAG diagnosis standard and TCM syndrome diagnostic standard;
- check to confirm diagnosis within a month before the inclusion experiment;
- agree to sign the informed consent;
- between 20 to 65 years old.

Exclusion Criterion

- patients with peptic ulcer, gastric mucosal dysplasia or pathological diagnosis of suspected malignant;
- patients with heart, brain, liver, kidney and hematopoietic system serious primary diseases, or mental problems;
- women with pregnancy or prepare to pregnant, breastfeeding women;
- allergic constitution and allergy to a variety of drugs;
- CAG heat and cold syndrome mingled with other syndromes.

Reagent and Instruments

MSTFA+1%TMCS（USA Pierce）, methoxamine hydrochloride（98% pure, Sigma-Aldrich）; alkane standard solutions（C8-C40, Sigma-Aldrich）; pyridines（≥99.8%GC, Sigma-Aldrich）; 1,2-13C2 tetradecanoic acid （USA Isotec）; methanol（pure chromatography，USA TEDIA）;

methyistearicacid（Sigma-Aldrich）；normal heptane（pure chromatography, Germany Merck）

GCMS solution V2.5 workstation、NIST lines library；High speed low temperature centrifuge (sigma company, 3k18)；low speed centrifuge (Shanghai Precise Operation Instrument Factory 80－2)；turbine mixer (Shanghai Xing Hang Industrial Company, TM-1).

Metabolomics Organization Sample Preparation and Extraction

All differentiations of the cases were taken by two attending physicians or higher position in the project team. Eligible people signed informed consent, and then fasted for 12 hours. When using electronic gastroscope (Olympus GIF - XQ260), take 2 biopsy blocks at sinuses ventriculi greater curvature or the same place of lesser curvature that 2~3cm from pylorus. One was sent for pathological examination; another was preserved in – 80 centigrade. The histopathological diagnosis consulted Chinese chronic gastritis consensus [11] and a person was designate to do pathological diagnosis. In Helicobacter pylori inspection WS was used for dyeing.

Take 20 mg tissue in 37 centigrade water bath to thaw for 15 minutes, add 800 μL single phase extraction liquid that containing myristate (2.5μL/ml) with stable isotope as internal label (water: methanol = 1:4), then grid. Vortex oscillate the mixture for 3 minutes then oximate in 20centigrade for 16 hours; Add 30μg BSTFA and vortex oscillate it for 3 minutes then derivative in 20 centigrade for 1 hour. Finally add 30μl external labeled stearic acid heptane fluid (30μg/ml), then vortex 3 minutes. What is acquired is the sample.

GC-MS Analysis

Gas phase condition: split ratio is 2:1; sample quantity is 0.5 u. Injection port temperature: 280centigrade; Interface temperature: 250 centigrade. Temperature program: 80centigrade, keep 2 minutes. Rise to 140centigrade at the speed of 10 centigrade /min, then rise to 240 centigrade at the speed of 4 centigrade/min, and then rise to 280 centigrade at the speed of 10centigrade/ min, and keep 3 minutes. Ion source temperature: 200 centigrade. Carrier gas: high-purity helium. Mass spectra conditions: carrier gas flow rate 1.0mL/min. Ionization mode: EI, electron energy is 70eV. Scanning range: 50～800m/z. According to the retention time of each peak in the GC-MS total ion chromatogram choose common peak, and obtain the peak area data of each peak and internal labeled peak, then use relative peak area (ratio with internal labeled peak) to represent the relative content of the metabolites.

Precision experiment: Take the same sample for 6 times continuously sampling and calculate the relative standard deviation (RSD) of the relative peak area.

Metabolite identification: Use NIST mass spectrum database to identify common endogenous metabolites. The identification result that matching degree > 80% is regarded as credible material.

Training

Project principal investigators provide professional training to the clinical centers before clinic, so that the researchers can fully understand clinical survey plan and its various standard operating procedures.

Qualifications of Principal Investigator

Investigator should have qualification above attending doctor, and relative fixed. The diagnosis of western medicine and TCM syndrome should be at least completed by an associate chief physician and a resident physician together, and clinical case report form to be filled by attending physician or above title.

Statistical Method

Use SPSS21.0 statistical software to carry on the calculation and adopt t-test, nonparametric test as analysis methods. Discrepancy P < 0.05 is regarded statistical significant.

Data mining and processing methods: Respectively use principal component analysis (PCA), cluster analysis and decision tree method to screen statistically significant characteristic metabolites that come from t-test and nonparametric test analysis in order to get CAG cold and heat syndrome classification model.

RESULTS

General Information

This experiment altogether collected 92 cases, 16 shedding cases, 23 eliminating cases. All cases come from inpatients of Dongzhimen hospital. Collection time is from 2012 to 2015. 28 cold syndrome cases, 25 heat syndrome cases; 15 male cases and 13 female cases in cold syndrome group, with an average age of 47; 13 male cases and 12 female cases in heat syndrome group, with an average age of 44 The gender, age baseline level of the two groups was consistent (P > 0.05); medical history and symptoms integral have no statistical difference. All selected people have signed informed consent, and by the

Ethics Committee of the Third Hospital of Peking University.

Characteristic Metabolites Screening Results

Extract 160 metabolites from 53 cases of chronic atrophic gastritis by NMR method. Various compounds account for different proportions in different patients. Table 1 lists 33 kinds of characteristic metabolite that frequency exceeds 50%.

Screening Results of Indicators Associated with Cold Syndrome and Heat Syndrome

Do t-test the above 33 kinds of compound to find specific ones. Results: three compounds that retention times are 21.83, 33.16, and 33.26 have significant difference ($P < 0.05$).

PCA and OPLS Pattern Recognition

Figure 1A and B shows the score diagram of principal component analysis (PCA) and orthogonal signal correction partial least squares (OPLS) pattern recognition. In the scored diagrams PC1 and PC2 indicate the first and the second principal components of main components, and reflects the distribution of cold syndrome and heat syndrome samples in the coordinate system that composed by PC1 and PC2. As can be seen from the diagram, CAG cold syndrome group and heat syndrome group can be better separated with the help of pattern recognition by PCA method (Fig. 1 A), each of the two samples has an outlier beyond 95% range (elliptical area in the figure).

Corresponding to score diagrams, the load diagram shows detected variable distribution. It is easy to identify the difference between the two groups of metabolites from NMR analysis, through the load diagram. as shown in Figure 1B: 33.26、33.16、21.83.

Table 1. List of 33 kinds of characteristic metabolite that frequency exceeds 50%.

retention time (s)	frequency (number of times)	rate (%)	retention time (s)	frequency (number of times)	rate (%)
18.79	47	0.88679	31.95	40	0.75472
33.16	46	0.86792	34.69	40	0.75472
9.19	45	0.84906	25.15	38	0.71698
10.9	45	0.84906	28.15	38	0.71698
12.5	44	0.83019	21.34	37	0.69811
21.72	44	0.83019	21.83	37	0.69811
33.78	43	0.81132	33.26	37	0.69811
8.73	42	0.79245	16.01	36	0.67925
10.14	42	0.79245	20.56	36	0.67925
14.77	42	0.79245	22.49	36	0.67925
23.53	42	0.79245	17.45	32	0.60377
19.79	41	0.77358	19.33	32	0.60377
24.15	41	0.77358	14.03	30	0.56604
7.56	40	0.75472	8.01	29	0.54717
9.61	40	0.75472	17.58	28	0.5283
11.98	40	0.75472	32.08	27	0.50943
15.29	40	0.75472			

Table 2. Evaluation Form of CAG Cold and Heat Syndrome Judgment Model Based on Decision Tree

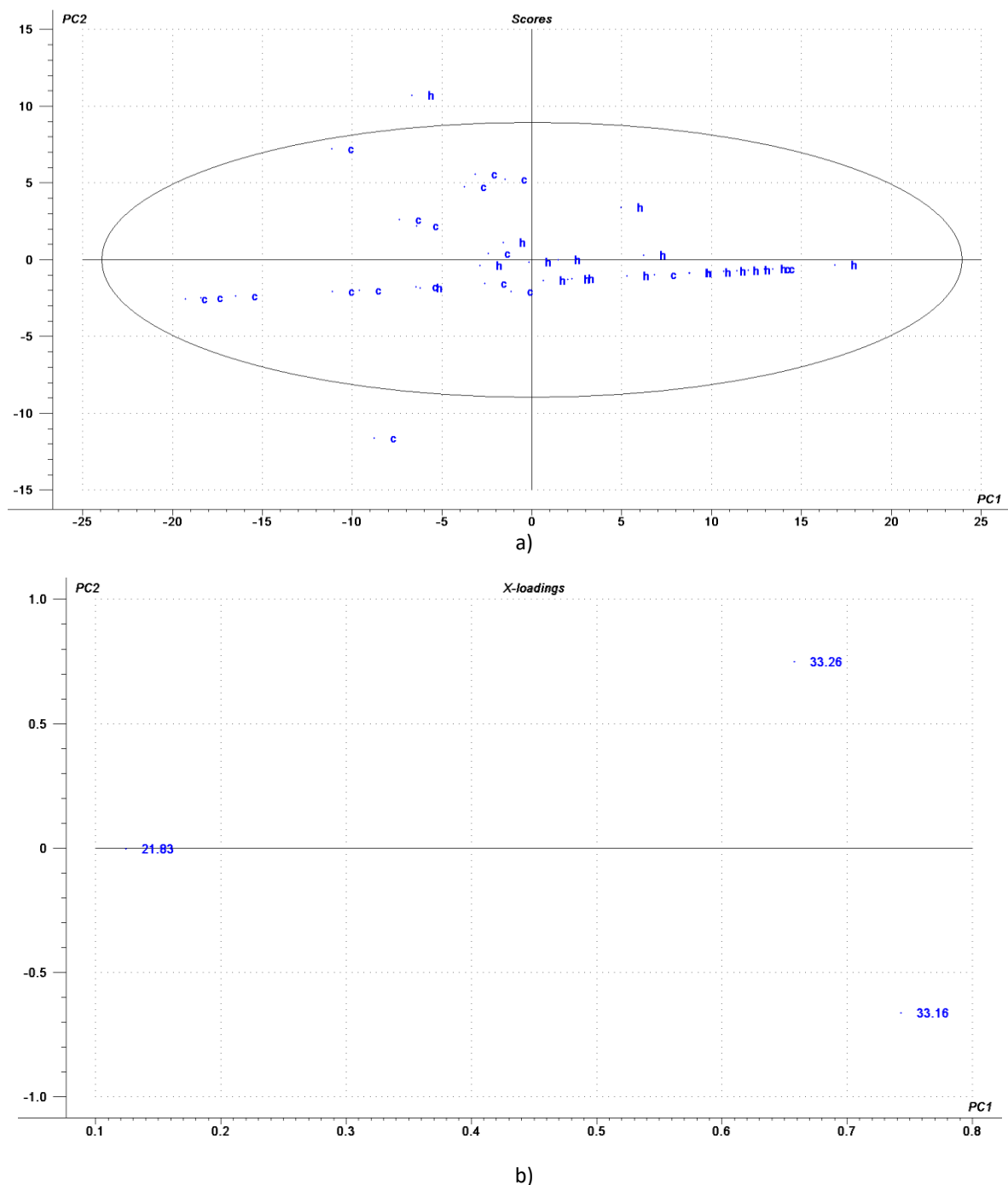| Category	Actual number of samples	Discernible number of samples	Negative detection accuracy (%)	Positive detection accuracy (%)	Detection accuracy (%)
Heat syndrome	36	25	18.5	81.5	81.5
Cold syndrome	40	28			

a)

b)

Fig. 1. a)PCA pattern recognition scoring diagram of CAG cold and heat syndrome; b) OPLS pattern recognition load diagram of CAG cold and heat syndrome.

Cluster Analysis

Take the characteristic metabolites screened out by T-test as variables to do cluster analysis on CAG cold and heat syndrome. The result is as shown in Figure 2.

Establishment of CAG Cold and Heat Syndrome Decision Tree Model

Adopt the significant compounds from T-test analysis as independent variables, and take "whether it is heat syndrome or not" as a dependent variable

into the decision tree model screening. Adopt Weka3.5.5J48 decision tree method to dig diagnostic rules, finally get decision tree classification model of CAG cold syndrome and heat syndrome.

There are five leaf nodes in the cold and heat syndrome decision tree classification model, and "TRUE" is on behalf of "heat syndrome" while "FALSE" is on behalf of "the cold syndrome. The number in parentheses represents the retention time of the compound. The model contains four kinds of compounds and their expression time was

33.16,21.83,33.26,34.69, which can be seen from Figure 3.

Evaluation of CAG Cold and Heat Syndrome Decision Tree Model

The testing accuracy of CAG cold and heat syndrome decision tree model is 81.5 %; Negative detection accuracy is 18.5 % and positive detection accuracy is 81.5 %.

Determine the Specific Metabolites

According to the retention time of the above-mentioned specific metabolites, find their corresponding possible metabolites from the information list of the metabolites, as shown in Figure 4A, 4B, 4C, 4D.
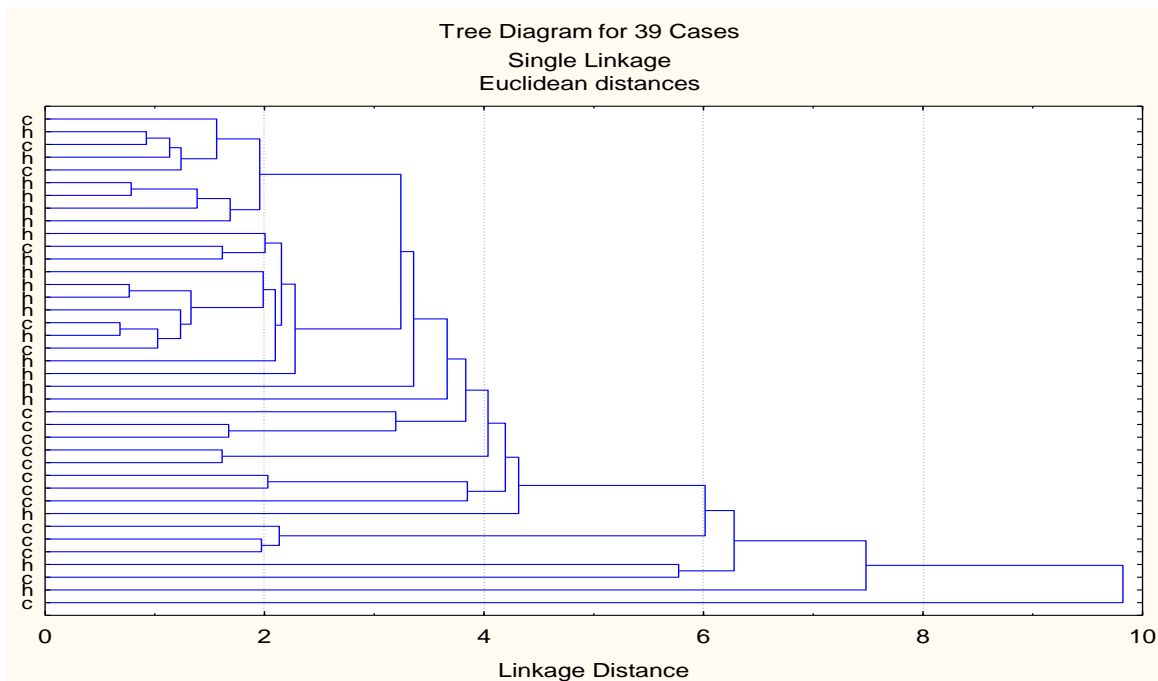
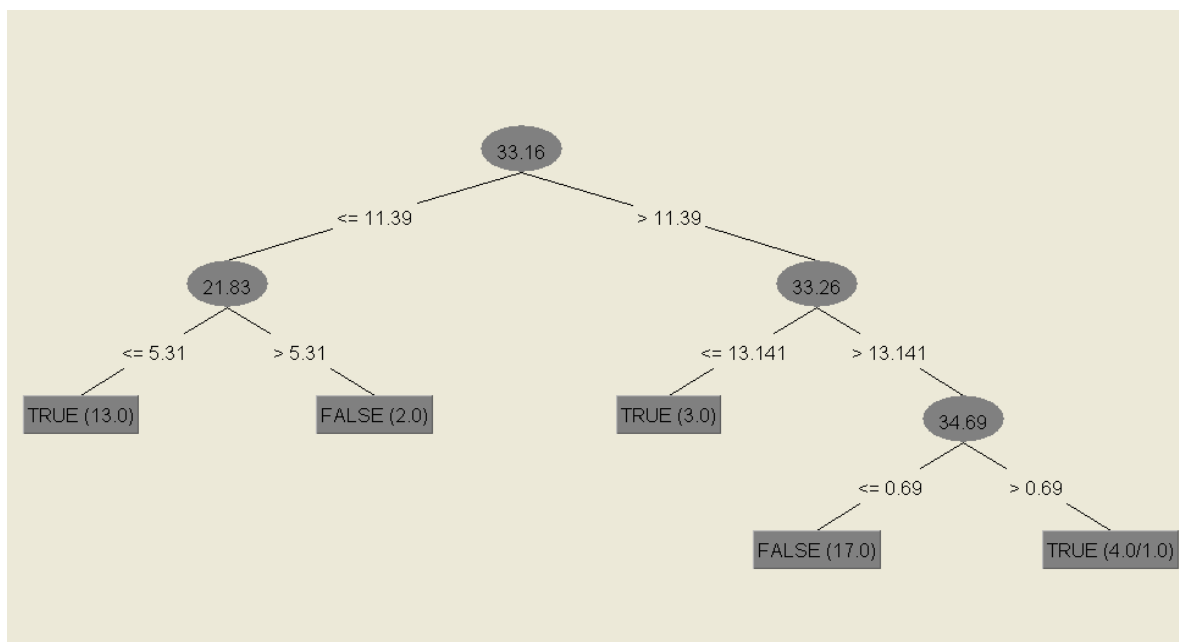**Fig. 2.** CAG cold syndrome and heat syndrome clustering analysis based on two characteristic metabolites

Fig. 3. CAG Cold and Heat Syndrome Judgment Schematic Diagram Based on the Decision Tree Method.
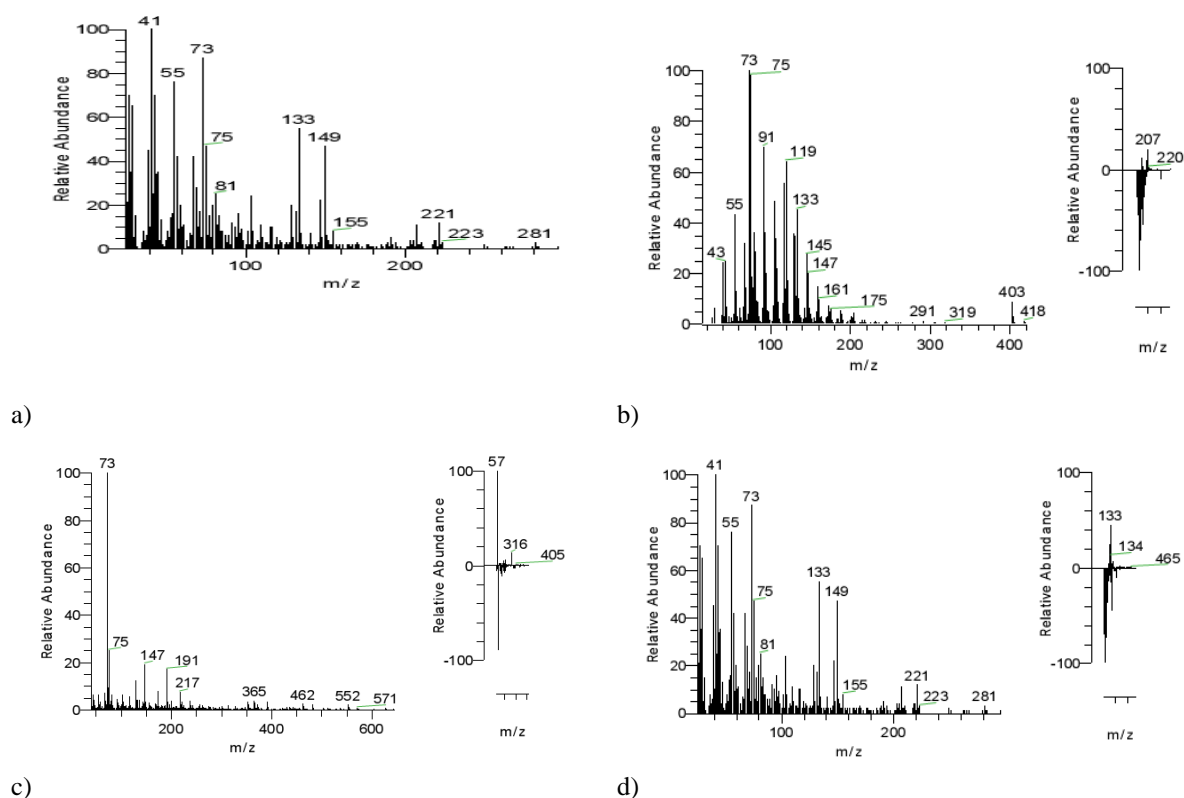
a)

b)

c)

d)

Fig. 4. a) 21.83; b) 33.16; c) 33.26; d) 34.69.

DISCUSSION

"Syndrome" is the starting point and the core of treatment based on syndrome differentiation. Syndrome refers to the synthetic reaction occurs when relationship between internal and external environment and all the systems disturb under the pathogenic factor function. It is a comprehensive diagnostic concept reflects the pathological elements of the etiology, pathogenesis, disease location, and disease potential at a certain stage. Because of syndrome holistic, dynamic, same syndrome existing in the different diseases and same disease belonging to different syndromes, and other characteristics, it is not possible to use a single indicator for qualitative, quantitative and positioning instructions. Accordingly, syndrome objective research uses comprehensive index and selects nonspecific index for specific combination to establish comprehensive, qualitative, quantitative, and positioning indicators which can reflects the nature of syndrome and distinguishes it from other syndromes. It is enormously significant on assisting the four methods of diagnosis and determining the syndrome diagnosis. Metabolomics possesses the "crowd", "group", "spectrum" integrated analysis function that can .reflect and solve these problems. It is able to test the blood or urine of patients at different times, analyze its metabolites, and determine syndrome corresponding metabolites, in order to provide a basis for objectification of syndrome differentiation and treatment [12].

This research used NMR technology to acquire CAG cold and heat syndrome metabolites, and analyzed CAG cold and heat syndromes' metabolomics product by principal component analysis, decision tree method and cluster analysis, preliminary got specific metabolite associated with CAG cold and heat syndrome and formed five judging ways of cold and heat syndrome with the decision tree model composed by 21.83、33.16、33.26、34.69, and the judgment accuracy was 81.5 %, and formed a clear, intuitive CAG cold and heat syndrome judgment model that may become the objective basis of CAG cold and heat syndrome differentiation and treatment. Therefore, the application that base on characteristics and advantages of metabonomics and combine data mining technology may provide new method for objective and dynamic observation of TCM syndrome.

REFERENCES

1. W.F. Zhu, *Beijing: People's Medical Publishing House,* **6**, 2 (2008).
2. X.Z. Yan, J.Y. Zhao, S.Q. Peng, M.Y. Liao, *J Magn Reson*, **2l**(2), 263 (2004)..
3. H. Zhu, H.R. Tang, X. Zhang, M.L. Liu, *Chem Bull*, **69**, l (2006).
4. J. LI, X.J. Wu, L.X. Liu, Y.J. Yuan, *Acta Pharm Sin*, **41**(1), 47 (2006).
5. Y. Guan, G. Liang, G.B. Martin, *BMC Genomics*, **18**(1), 64 (2017)
6. M. Ghoneum, M. Matsuura, M. Braga, *Int J Oncol*, **33**(3), 533 (2008).
7. S. Li, Z.Q. Zhang, L.J. Wu, X.G. Zhang, Y.D. Li, Y.Y. Wang, *IET System Biology*, **1(1)**, 51 (2007).
8. Wu X. Wu, Y. Song, W. Liu, *Cell Death Discov*, **30**, 3 (2017).
9. T.T. Deng, *Shanghai Sci Tec Publishers*, 97 (1984).
10. N.H. Pang, Z.X. Fan, S.L. Niu, *Chinese J Integr Tradit Wes Med*, **7**(11), 652 (1987).
11. Z.Y. Hu, *Gastroenterology*, **1l**(11), 674 (2006).
12. S.Y. Sun, L. Jin, *Chinese J Inf TCM*, **15**(4), 3 (2008).