RESEARCH ARTICLE

Metabolomics Investigation of Cutaneous T Cell Lymphoma Based on UHPLC-QTOF/MS

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Abstract

Objectives: The identification of cutaneous T cell lymphoma (CTCL) biomarkers may serve as a predictor of disease progression and treatment response. The aim of this study was to map potential biomarkers in CTCL plasma. Design and Methods: Plasma metabolic perturbations between CTCL cases and healthy individuals were investigated using metabolomics and ultrahigh performance liquid chromatography–quadrupole time-of-flight mass spectrometry (UHPLC-QTOF/MS). Results: Principal component analysis (PCA) of the spectra showed clear metabolic changes between the two groups. Thirty six potential biomarkers associated with CTCL were found. Conclusions: Based on PCA, several biomarkers were determined and further identified by LC/MS/MS analysis. All of these could be potential early markers of CTCL. In addition, we established that heparin as anticoagulant has better pre-treatment results than EDTA with the UHPLC-QTOF/MS appraoch.

Keywords: Cutaneous T cell lymphoma - metabolomics - biomarkers - UHPLC-QTOF/MS

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Introduction

Cutaneous T cell lymphoma (CTCL) is the most common primary malignant T cell lymphoma of the skin, presenting as erythema, plaques, tumors or erythroderma. Incidence is 1/10,000 people per year. Although, the actual incidence of the disease may be underestimated due to different diagnostic criteria (Chuang et al., 1990; Weinstock et al., 1999). The median onset age of CTCL has been reported as 57 years (Kim et al., 2003). But a report from China states the median onset age as being 47.5 years before 2000, and 34 years from 2001-2008, showing the onset of CTCL tends to be in the young (Li et al., 2008). Mycosis fungoides is the most frequent type of CTCL, which has an indolent course initiating as erythema, plaques for years and involving lymph nodes or visceral organs as the disease advances. The aggressive type of CTCL has a poor prognosis (Regina et al., 2002). The etiology of CTCL remains poorly understood, and occupational exposure, virus infection and genetic mutation have been proposed as etiological factors. The aberrant expression and function of the transcriptional factors and regulators of signal transduction have been reported in CTCL (Yin et al., 2013). It has been hypothesized that a dysfunctional regulation of small molecules plays a key role in the malignant formation. The occurrence and development of CTCL is a dynamic process regulated by multiple genes, ultimately leading to tumor metabolic changes. The use of metabolomics to study different blood samples from CTCL patients and healthy volunteers, by searching for small and diagnosis-related molecular biomarkers, may offer the opportunity for early diagnosis of CTCL. In this study, we aim to differentiate the expression of metabolic molecules in CTCL and map the potential biomarkers in CTCL plasma.

LC/MS has been widely applied in the analysis of biological sample according to Kunnathur et al. (Kunnathur et al.,2013).In metabolomics, tandem mass spectrometry was often used to detect as many metabolites as possible. In addition, TOF mass analyzer can provide a more accurate molecular weight of metabolites compared to the others. Ultrahigh performance liquid chromatography (UHPLC)-quadrupole time-of-flight mass spectrometry (QTOF/MS) was employed to profile the plasma of patients with CTCL. Differences in metabolomics data from the two groups were characterized using principal components analysis (PCA). Based on pattern recognition results, we aimed to establish a diagnosis model and explore the potential metabolic biomarkers for early diagnosis and staging in gastric cancer.

Sample pretreatment process is a critical factor for the success of metabolomics research studies. Therefore the major steps in sample pretreatment including plasma extraction method, stability of plasma samples at 25°C and anticoagulation in sample collection were investigated.

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Materials and Methods

Patient recruitment

This prospective study was approved by the Ethics Committee of Ruijin Hospital, Shanghai Jiaotong University. Informed consent was obtained from all participants. CTCL plasma was obtained from 35 patients and matched plasma taken from healthy volunteers. The skin biopsy was examined by histopathological and immunohistochemical stain and T cell receptor gene rearrangement. The diagnosis was made by two experienced dermatopathologists. A computerized tomography (CT) scan and lymph node ultrasound examination were used to determine the tumor stage. (Xia et al., 2013) All patients were evaluated by the same dermatologist.

Sample collection

Venous blood was collected from 30 control subjects and 35 CTCL patients without medication. All the samples were then transferred into heparinized tubes and immediately centrifuged at 12,000 rpm for 10 minutes. Fresh plasma was frozen in liquid nitrogen during operation, then stored at -80°C until processing. To a 150 µL aliquot of plasma samples, 450 µL of methanol was added for protein precipitation. After centrifugation at 12,000 rpm for 10 minutes, an aliquot of 10 µL supernatant was injected for UHPLC/MS analysis.

UHPLC-QTOF/MS analysis

Plasma samples were analyzed by an Agilent UHPLC-QTOF/MS (Agilent Corporation, Santa Clara, CA, USA). Chromatography was performed on a 100 x 2.1 mm ACQUITY UPLC® HSS T31.8 µm column (Waters Corporation, Milford, MA, USA). The mobile phase, with 0.3 ml/min flow rate, consisted of solvent 0.1% formic acid (Sigma-Aldrich, Germany) and acetonitrile containing 0.05% formic acid. Gradient elution was performed and the program is shown in Table 1.

Mass spectrometric detection was carried out on an Agilent 1260 Infinity series mass spectrometer (Agilent Corporation). The electrospray ionization source was set in positive and negative modes, respectively. Instrument parameters were set as follows: capillary voltage 3.5 kV, gas temperature 350°C and desolvation temperature 350°C. Nitrogen was used as desolvation and cone gas with a flow rate of 10 L/min, respectively. Full scan mode was employed in the mass range of 100–1000 amu. In the MS/MS experiments, argon was used as collision gas and collision energy was set according to the construction of metabolites. Data were collected in centroid mode.

Table 1. The Program of Gradient Elution

	Time(min)	A%	B%	Flow(ml/min)
Positive mode	0	2	98	0.3
	2	2	98	0.3
	12	95	5	0.3
	22	95	5	0.3
Negative mode	0	2	98	0.3
	2	2	98	0.3
	12	95	5	0.3
	22	95	5	0.3

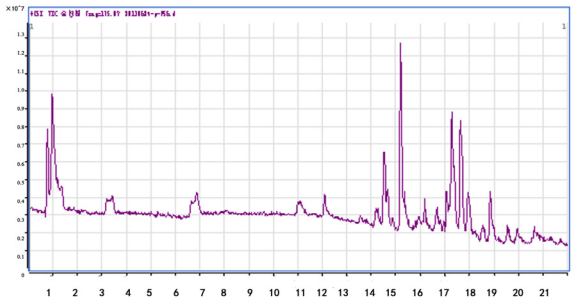

Figure 1. A Typical Ultrahigh Performance Liquid Chromatography-Quadrupole Time-of-Flight Mass Spectrometry (UHPLC-QTOF/MS) Total Ion Chromatogram of Study Sample

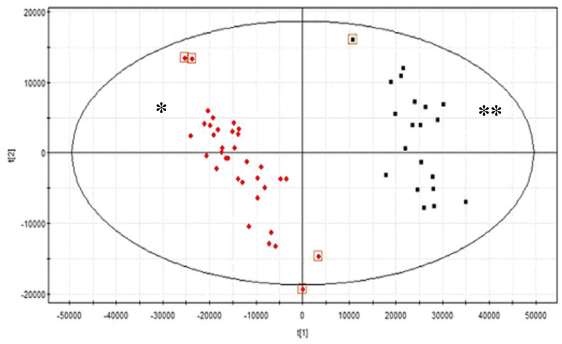

Figure 2. Partial Least Squares-Discriminate Analysis (PLS-DA) of All Data. (*control samples, **study samples)

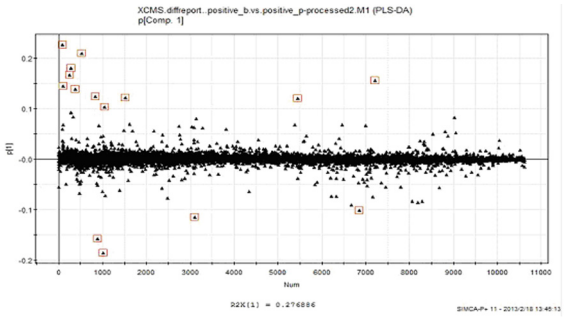

Figure 3. Scatter Plots of All Endogenous Metabolites

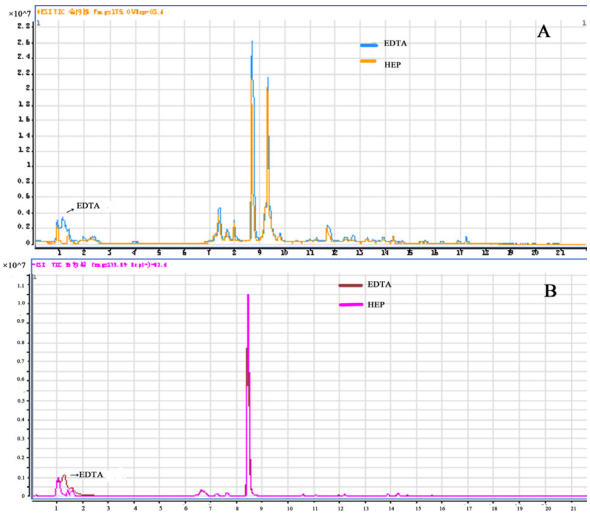

Figure 4. Total Ion Chromatogram of Globulin with EDTA and Heparin in the Positive(A)/Negative(B) mode

Table 2. Results of comparison between gadobenate dimeglumine with the golden standard (95%CI)

Positive Mode				
M+H m/z	Retention time	Metabolites	MW of metabolites+H	MW of metabolites
162.12	0.93	Nicotine imine	162.1151	161.1079
496.35	19.34	LysoPC (16:0)	496.3398	495.3325
214.92	0.76	1, 1-Dibromo-2-propanone	214.8702	213.8629
		Edetic Acid	293.0979	292.0907
120.08	1.87	L-Threonine	120.0655	119.0582
		L-Homoserine	120.0655	119.0582
		L-Allothreonine	120.0655	119.0582
		Oxoglutaric acid	147.0288	146.0215
175.02	0.59	trans-Aconitic acid	175.0237	174.0164
		Dehydroascorbic acid	175.0237	174.0164
		cis-Aconitic acid	175.0237	174.0164
137.05	1.3	Hypoxanthine	137.0458	136.0385
243.29	15.45	1-Hexadecanol	243.2682	242.261
Negative Mode				
M-H m/z	Retention time	Metabolites	MW of metabolites+H	MW of metabolites
344	1.38	Guanosine 2',3'-cyclic phosphate	344.04016	345.04743
297.16	22.16	Minaprine	297.17209	298.17936
293.18	26.07	17-Hydroxylinolenic acid	293.21222	294.2195
		13-OxoODE	293.21222	294.2195
		9(10)-EpODE	293.21222	294.2195
313.05	1.25	Beta-D-Glucopyranuronic acid	313.05651	314.06378
		1-Salicylate glucuronide	313.05651	314.06378
		Geranyl-PP	313.06115	314.06843
		5'-Phosphoribosyl-N-formylglycinamide	313.04424	314.05152
		7,8-Dihydropteroic acid	313.10546	314.11274
300.01	1.38	N-Acetyl-D-galactosamine 1-phosphate	300.04899	301.05627
		N-Acetyl-D-Glucosamine 6-Phosphate	300.04899	301.05627
		N-Acetyl-D-mannosamine 6-phosphate	300.04899	301.05627
315.05	1.28	Olivin	315.08741	316.09469
298.16	22.07	Hydrocodone	298.14487	299.15214
174.96	0.89	Ascorbic acid	175.02481	176.03209
		D-Glucurono-6,3-lactone	175.02481	176.03209
565.34	21.95	Nigroxanthin	565.40511	566.41238
589.34	22.06	D-Urobilinogen	589.30316	590.31044
554.36	23.9	Pseudoecgonine	554.3083	185.10519
409.24	25.68	LPA(16:0/0:0)	409.23606	410.24334
		LPA(0:0/16:0)	409.23606	410.24334
195.81	1.2	Ubiquinone 6	195.80389	590.43351

Data processing and identification

LC/MSD ChemStation software (Agilent, Shanghai, China) was used for autoacquisition of LC total ion chromatograms (TICs) (Figure 1) and fragmentation patterns. The accurate mass number of every peak was available by XCMS-Online.

Compounds were mainly identified by searching the Human Metabolome Database (www.hmdb.ca) and by matching m/z (accurate mass number). Biomarkers were identified using biochemical databases such as KEGG and METLIN.

Statistical analysis

Qualitative analysis of MassHunter Acquisition Data was used to analyze the experimental data. Results are presented as means ±standard deviation (SD). Data that were not normally distributed were logarithmically transformed to obtain normal distribution before analysis. Continuous variables were analyzed by one-way ANOVA with Tukey's test. $P>0.05$ was considered to be statistically significant. A logistic regression analysis was performed to obtain the score of significant difference between CTCL and control samples.

Results and Discussion

Plasma pretreatment

Plasma sample collection and handling procedures are critical for successful metabolomics research studies (Yin et al., 2013). Firstly, in the sample collection step, commonly used anticoagulants such as ethylenediaminetetraacetic acid (EDTA) and heparin were investigated. In order to avoid plasma matrix interference, globulin samples were added with EDTA and heparin, respectively, to analyze the matrix effect of anticoagulants. The results showed that in either positive or negative mode, an EDTA sample has an impurity peak at 1 min compared with heparin. Heparin was therefore chosen as anticoagulant in our plasma sample collection. Protein precipitation by methanol or acetonitrile was tested respectively.

Table 3. Stability and Precision

Stability within day				
Peak No.	m/z	Compound	RSD%(average) Retention time	n=5 Peak area
1	137.0458	Hypoxanthine	0.577(1.458)	6.68
2	242.261	1-Hexadecanol	0.2249(11.119)	3.48
3	496.348	LysoPC(16:0/0:0)	0.254(14.411)	2.29
4	524.3792	LysoPC(18:0/0:0)	0.213(15.635)	8.65
Precision results				
Peak No.	m/z	Compound	RSD%(average) Retention time	n=5 Peak area
1	137.0458	Hypoxanthine	0.440(1.356)	5.33
2	242.261	1-Hexadecanol	0.09(11.190)	5.96
3	496.348	LysoPC(16:0/0:0)	0.055(14.332)	3.42
4	524.3792	LysoPC(18:0/0:0)	1.570(15.502)	4.22

Optimization of UHPLC/MS method

Different mobile phase systems were investigated, such as formic acid-methanol and formic acid-acetonitrile. Considering the separation and running time, the mobile phase adopted in our study consisted of solvent 0.1% formic acid and acetonitrile containing 0.05% formic acid with gradient elution program.

Precision and stability

The applied method was validated prior to the analysis of the experimental samples, including the precision of injection, the within-day stability and the repeatability of sample preparation. Extracted ion chromatographic peaks of four ions (m/z 137.0458, 1.356 min; m/z 242.2610, 11.190 min; m/z 496.3480, 14.332 min; m/z 524.3792, 15.502 min) distributed in different spectrum regions and retention time were selected for the method validation. Compared with the standards (retention time and m/z), four compounds were identified among the 36 potential biomarkers: Hypoxanthine, 1-Hexadecanol, LysoPC (18:0/0:0) and LysoPC (16:0/0:0). The relative standard deviations (RSDs) of peak intensities and retention time for the selected ions in pooled plasma sample were calculated.

UHPLC/MS metabolite profiles

The preparation of plasma samples for metabolic profiling analysis by UHPLC/MS involved a protein precipitation step to extract low-molecular weight compounds and remove the large amounts of proteins that would otherwise interfere with the UHPLC/MS analysis. More quantitative information was obtained from the positive ion mode than that collected under the negative ion mode, so that molecular ions (M+H)+ accounted for the majority of the mass spectrum. Figure 1 shows the representative positive base peak intensity.

Analysis of UHPLC/MS data

In order to find the metabolites with a significant change (i.e., potential biomarkers), partial least squares-discriminate analysis (PLS-DA) was constructed using the metabolite intensities as variables. As a classic unsupervised method (no prior knowledge concerning groups or tendencies within the data sets was necessary) for pattern recognition, PLS-DA was expected to pick out distinct variables as potential biomarkers through statistical protocols. The score plot for PLS-DA of control and CTCL patients is shown in Figure 2. Two groups were separated with a clear border. The results showed that the method could find potential biomarkers and distinguish CTCL patients from healthy people.

Biomarker identification

In the LC/MS TICs of samples from the study and control groups, the majority of the peaks were identified as endogenous metabolites based on the Human Metabolome Database, including amino acids, organic acids, inorganic acids, carbohydrates, fatty acids, aldehydes, amines, amides, polyols and pyrimidines (Wu et al., 2010). There were about 600 signals obtained.

SIMCA-*P* was used to find metabolites whose contents were significantly different between CTCL and control samples (Figure 3). According to the analysis results, we chose absolute values with $P>0.05$ as potential biomarkers (Table 2).

Study of stability and precision

Extracts of plasma samples were stored at 25°C and injected at 0, 4, 8, 12 and 24 hours, respectively. The RSDs of peak intensities and retention time for the selected ions in pooled plasma sample were calculated (Table 3) (Yang et al., 2013).

Precision of injection was carried out by the continuous detection of five injections of the same standard sample which was considered a potential biomarker. RSDs ranged from 0.09-1.6% for retention time and from 3.5-6.0% for peak intensity. The results are shown in Table 3.

Study of matrix effect

Globulin samples were added with EDTA and heparin respectively to analyze the matrix effect of anticoagulants (Figures 4) (Yang et al., 2013). Either in positive or negative mode, EDTA sample has an impurity peak at 1 min compared with heparin. Therefore, we chose heparin as the anticoagulant based on its better pre-treatment results.

Acknowledgements

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