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Metabolomics analysis of cervical cancer, cervical intraepithelial neoplasia and chronic cervicitis by ^{1}H NMR spectroscopy

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Summary

Metabolomics profiles of serum samples from women with chronic cervicitis, cervical intraepithelial neoplasia (CIN), and cervical cancer were characterized by proton nuclear magnetic resonance (^{1}H NMR). These spectral profiles were subjected to partial least-squares discriminant analysis (PLS-DA), and good discriminations between cancer and non-cancer groups (chronic cervicitis and CIN) were achieved by multivariate modeling of serum profiles. The main metabolites contributing to these discriminations, as highlighted by multivariate analysis and confirmed by spectral integration, were formate, tyrosine, β-glucose, inositol, glycine, carnitine, glutamine, acetate, alanine, valine, isoleucine, and very-low-density lipoprotein (VLDL). Metabolomics analysis for chronic cervicitis, CIN, and cervical cancer is significant, which give a systemic metabolic response of these female diseases. The systemic metabolic response may be used to identify the potential biomarkers for the diseases.

Key words: Metabolomics; Cervical cancer; Cervical intraepithelial neoplasia; Chronic Cervicitis; ^{1}H NMR spectroscopy; PLS-DA.

Introduction

Cervical cancer is a kind of malignant tumor and represents a significant disease burden. This cancer is serious harm for the majority of women's health, and its incidence rate is second only to breast cancer for women [1-5]. With the work of mass screening and treatment of cervical cancer, the incidence rate of cervical cancer in the world has declined in near 40 years. However, due to the worse environmental pollution and personal hygiene practices, the incidence trend of the patients with cervical cancer was younger, and the incidence of precancerous was increased [6]. Cervical intraepithelial neoplasia (CIN), a common type of precancerous disease of cervical cancer, has significantly increased the risk of cervical cancer, and the same is true of chronic cervicitis for CIN. Therefore, early diagnosis and detection of cervical cancer is more critical for much higher survival rate. Currently, cervical cancer can be characterized by the Papanicolaou (Pap) smear test [7, 8], thin layer of liquid crystal cytology [9-11], and colposcopy [12]. However, these cytological screenings have inherent defects that produce false-negative/-positive results and subjective judgment [13, 14], which tend to result in insufficient diagnostic sensitivity and specificity. To improve survival rate, more sensitivity and specificity method should be developed for early detection and treatment of cancer successfully, which may identify novel biomarkers and molecular targets. Proteomics and metabolomics are powerful analytical tools that can provide worthy information on complex biological samples. Proteomics has more advantages of analyzing various biological samples, and detecting some differently expressed proteins. Serum protein profiling of patients with cervical cancer has been screened by using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry [15], and the differentially expressed proteins between cervical cancer and healthy samples have been discovered.

Metabolomics is the study of metabolic processes in biological systems, which goals are to identify metabolic biomarkers or predictors associated with a specific biochemical event and its effect. Some work has been reported regarding such applications in this field [16, 17]. Ultra-performance liquid chromatography quadrupole time of flight mass spectrometry (UPLC/QTOF/MS) [18] has been used for identifying metabolic biomarkers to diagnose epithelial ovarian cancer, and the metabolism of diabetic urine samples [19] has been studied by liquid chromatography–mass spectrometry (LC/MS) and ^{1}H Nuclear magnetic resonance (NMR) spectroscopy method.

NMR spectroscopy is one of the main research methods of metabolomics [20-22]. Recent literature also used ^{1}H NMR-based metabolomics to detect epithelial ovarian cancer [23]. The results of previous study by using ^{1}H NMR revealed the relationships of choline:creatine ratio, and lactate levels with cervical cancer [24].

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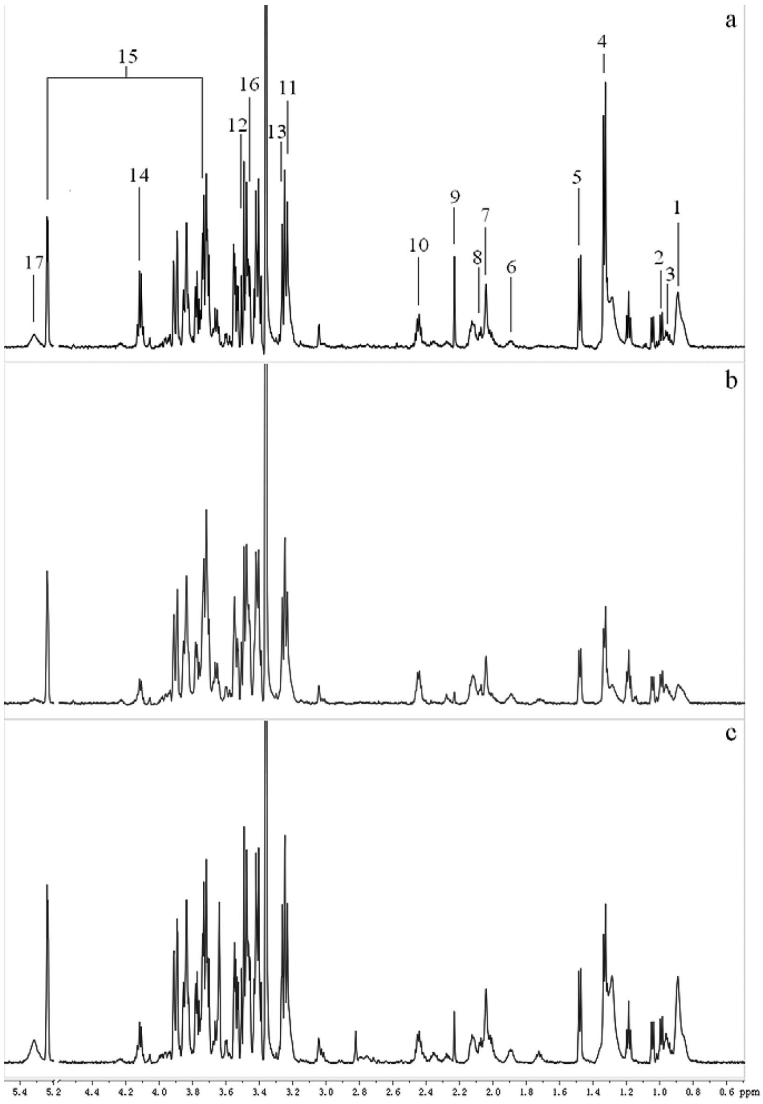

Figure 1. — Representative 600 MHz ^{1}H NMR spectrogram of serum from δ5.55 to δ0.5. (a) patients with chronic cervicitis, (b) CIN, and (c) cervical cancer. Only significant metabolites have been labeled in (a). 1: very low density lipoprotein (VLDL), 2: isoleucine, 3: valine, 4: lactate, 5: alanine, 6: acetate, 7: acetylcysteine, 8: glutamine, 9: acetone, 10: carnitine, 11: choline, 12: glycine, 13: inositol, 14: creatine, 15: β-glucose, 16: α-glucose, 17: unsaturated lipid, 18: tyrosine, 19: histidine, 20: formate.

Recent researches have mainly focused on cytology of cervical cancer in order to understand the metabolic processes and mechanisms during the development of cancer [25, 26], and during the radiotherapy of cervical cancer, the changes had been measured polarographically [27, 28]. Magic angle spinning magnetic resonance spectroscopy was applied to characterize cervical cancer tissue [29, 30]. There are also some literatures that researched the phospholipid metabolism by ^{31}P NMR spectroscopy [31]. However, these papers of tissue studies may not be non-destructive for patients, analyzing samples of serum or urine could be given more attention [32-34]. Furthermore, the metabolomic analysis of the serum of patients with cervical cancer and its precancerous diseases is not enough by far.

In this study, serum samples from patients with chronic cervicitis, CIN, and cervical cancer were subjected to metabolomic analyses by ^{1}H NMR spectroscopy, followed by partial least-squares discriminant analysis (PLS-DA) to analysis the serum metabolites in the three groups and identify the potential biomarkers.

Materials and Methods

Sample collection

All the diagnoses of chronic cervicitis, CIN, and cervical cancer were confirmed by histopathology. A written informed consent was obtained from all patients, voluntarily. Number of patients with chronic cervicitis was 22, and the average age was 31 years (22-43 years). Number of patients with CIN was nine, and the average age was 33 years (24-43 years). Number of patients with cervical cancer was 18, and the average age was 40 years, (35-46 years), and the total number of patients was 49. All samples were collected from 2010 to 2012. The blood sample was taken at around the same time prior to breakfast for each patient. The serum was obtained by centrifugation of the blood sample in tube at 1,300 g for 20 minutes at 4 ˚C. The treated serum samples were stored at -60 ˚C until NMR analysis.

Preparation of serum samples for ^{1}H NMR spectroscopic analysis

The serum samples were prepared according to literature [21]. In brief, the frozen serum samples were thawed prior to use. Then the serum samples were prepared for NMR analysis by mixing 200 μL of serum with 400 μL D_2O. The serum-D_2O mixture was then centrifuged at 12,000 g for ten minutes. The clear supernatant (550 μL) was then transferred to a five-mm NMR tube, which was used for NMR analysis later.

Acquisition ^{1}H NMR spectroscopy of serum

NMR data were acquired on a Varian Unity Inova 600 spectrometer operating at 600.00 MHz ^{1}H observation frequency. Water signals and broad protein resonances were suppressed by a combination of presaturation and the Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence. Typically, 64 free induction decays (FIDs) were collected into 32 k data points using a spectral width of 8,000 Hz and acquisition time of two seconds and a relaxation delay of four seconds. Spectroscopy were acquired at 298 K. The data were zero filled, and the FIDs were multiplied by an exponential weighting function equivalent to a line broadening of 0.3 Hz prior to Fourier transformation (FT). The assignments of ^{1}H NMR spectroscopy were made, which referenced to published literatures [16, 34, 35].

^{1}H NMR spectroscopy were processed and corrected for phase and baseline with MestReNova 6.1.0.6224 software. Chemical shifts were referenced to the anomeric proton of lactate at δ1.336. The region 5.20-4.60 ppm was removed in order to avoid the effects of water suppression. The spectra of δ_H=9.0 to 0.02 ppm were put into 1,675 integrated regions of 0.005 ppm, and the data needed to be normalized by peak area. The data were then imported into Microsoft Excel.

Statistical analysis of metabolites

The normalized integral values were imported into the SIMCA-P+11.5 software as variables for the multivariate pattern recognition analysis. Partial least-squares discriminant analysis (PLS-DA) method was used for the class discrimination and the identification of metabolites. The normalized NMR data were then subjected to classical statistical analysis using SPSS 19.0 software. The statistically significant result was indicated by $p < 0.05$. Student's *t*-test also gave a significant difference of the metabolite content for the different stage of these diseases.

Results

^{1}H NMR spectroscopic analysis of serum

The ^{1}H NMR spectra of chronic cervicitis, CIN, and cervical cancer are shown in Figure 1, respectively. According to the published literatures, some metabolites in serum of patients with cancer or inflammation may be metabolic abnormalities, such as lactate, serine, alanine, glycine, phenylalanine, and glucose. By analyzing the different spectra of serum, it would be found that there were some changes of metabolites existed between cervical cancer and chronic cervicitis or CIN.

Difference between cervical cancer and its precancerous diseases using pattern recognition analysis

For obtaining an objective statistical estimation, PLS-DA for a model discriminating was used between the samples from patients with chronic cervicitis, CIN, and cervical cancer (shown in Figure 2). Figure 2a shows that the PLS-DA

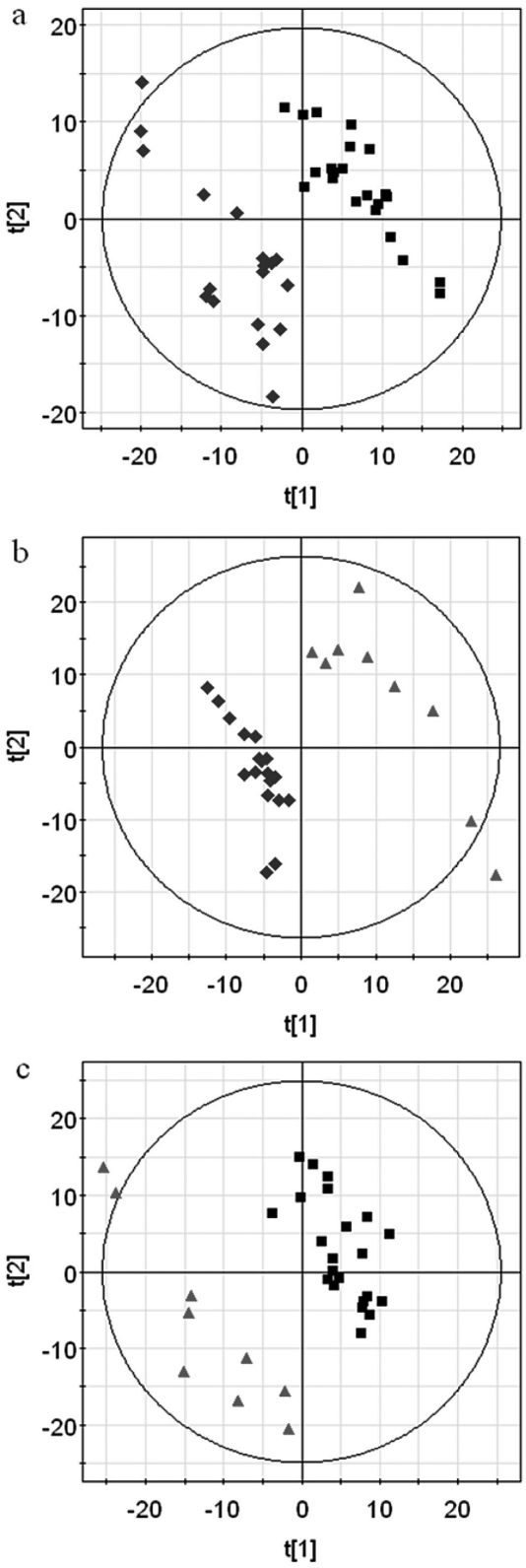

Figure 2. — PLS-DA models based on ^{1}H NMR spectroscopy of serum of patients. (a) discrimination between cervical cancer and chronic cervicitis. (b) discrimination between cervical cancer and CIN. (c) discrimination between chronic cervicitis and CIN. ■ chronic cervicitis, ▲ CIN, ◆ cervical cancer.

Table 1. — *Serum metabolites of patients with chronic cervicitis, CIN, and cervical cancer.*

Metabolites	Chemical shift	CIN	chronic cervicitis		Possible biochemical origin(s)
		$p< 0.005$ FC[a]	$p< 0.005$	FC[a]	
VLDL	0.81(m), 0.88(m), 1.26(m), 1.57(m), 2.09(m)	—	+1.049	0.001	+2.470 lipid metabolism
Isoleucine	0.93(t), 1.00(d)	—	-1.025	0.026	-1.364 energy metabolism
Valine	0.98(d), 1.04(d)	—	-1.043	0.013	-1.355 TCA cycle
Lactate	1.33(d), 4.11(q)	—	+1.172	—	-1.026 energy metabolism
Alanine	1.47(d), 3.77(q)	0.024	+1.803	0.036	+1.635 energy metabolism
Acetate	1.94(s)	—	-1.580	0.016	+1.888 energy metabolism; pyrimidine and amino acid degradation
Acetylcystenine	2.07(s)	—	-1.089	—	-1.086 energy metabolism
Glutamine	2.10(m), 2.13(m)	0.030	-1.620	0.023	-1.433 TCA cycle
Acetone	2.22(s)	—	-4.055	—	+1.362 energy metabolism; lipid metabolism
Carnitine	2.46(dd)	0.015	-1.640	0.018	-1.462 lipid metabolism
Creatine	3.03(s), 3.92(s)	—	+1.180	—	+1.207 serinolysis
α-glucose	3.24(dd), 3.49(t), 4.59(d)	—	+1.199	—	-1.057 glycolysis
Choline	3.25(s)	—	-1.022	—	+1.144 serinolysis; phospholipid metabolism
Inositol	3.27(t), 3.56(dd), 3.65(dd)	0.028	-1.552	0.002	-1.421 lipid metabolism
Glycine	3.58(s)	—	+1.474	0.041	+2.059 serinolysis
β-glucose	3.72(dd), 5.23(d)	0.039	-1.429	0.002	-1.729 glycolysis
Unsaturated lipid	5.26(m), 5.32(m), 5.37(m)	—	+1.036	—	+1.329 lipid metabolism
Tyrosine	6.88(d), 7.18(d)	—	-1.561	0.023	-1.075 energy metabolism; TCA cycle
Histidine	7.06(s), 7.73(s)	—	+1.204	—	-1.217 TCA cycle
Formate	8.45(s)	0.012	-1.250	0.019	-1.276 energy metabolism; pyrimidine and amino acid degradation

[a] FC: fold change between cervical cancer and its precancerous diseases. Positive sign indicates a higher level in cancer group and negative sign indicates a lower level.

scatter plot of the cervical cancer and chronic cervicitis patients (R^2X=0.225, R^2Y=0.992, Q^2=0.527). Obviously, Figure 2b shows the scatter plot of cervical cancer and CIN patients (R^2X=0.147, R^2Y=0.932, Q^2=0.168) which were located in different clusters. The PLS-DA scatter plot for chronic cervicitis and CIN patients (Figure 2c, R^2X=0.142, R^2Y=0.911, Q^2=0.260) also shows clear separation and discrimination. These results demonstrated a different metabolic profile in patients with cervical cancer and non-cancer groups, which indicated that there were some serum metabolites contributing to these discriminations. Furthermore, there were several points far away from the center for the serum samples; it may be attributed to a slightly longer period of sample storage [36, 37].

According to the statistical analysis using PLS-DA as unsupervised and supervised methods, respectively, the samples of patients with cervical cancer, CIN, and chronic cervicitis were all scattered into their regions. Figure 2 represented good discrimination of the cancer from chronic cervicitis and CIN with the pattern of metabolites and these results suggested that patients with cervical cancer have a specific profile which was different from patients with chronic cervicitis and the CIN.

Loading plots calculated from the PLS-DA models were to identify metabolites for different models. There were 20 metabolites can be used to separate chronic cervicitis, CIN, and cervical cancer, which are shown in Table 1. The main metabolites contributing to these discriminations were formate, histidine, tyrosine, unsaturated lipid, β-glucose, glycine, inositol, choline, α-glucose, creatine, carnitine, acetone, glutamine, acetylcystenine, acetate, alanine, lactate, valine, isoleucine, and VLDL.

When the data were subjected to classical statistical analysis using SPSS, the statistically significant results were chosen by p<0.05. Then, there were 12 metabolites were considered to indicate statistically significant results, which were formate, tyrosine, β-glucose, inositol, glycine, carnitine, glutamine, acetate, alanine, valine, isoleucine, VLDL. Compared with chronic cervicitis and CIN, the levels of VLDL, alanine and glycine were increased in the samples of patients with cervical cancer, whereas the levels of isoleucine, valine, glutamine, carnitine, inositol, β-glucose, tyrosine and formate were reduced. However, compared with cervical cancer, acetate had higher concentration in CIN, and lower concentration in chronic cervicitis.

Because alanine, glutamine, carnitine, inositol, β-glucose and formate were both significant different between cervical cancer and its precancerous diseases (CIN and chronic cervicitis), the altered expressions of these six metabolites were calculated and illustrated in Figure 3. And VLDL, inositol and β-glucose were much more different as their p<0.01.

Discussion

Cervical cancer patients were characterized as possessing relatively lower abundance of isoleucine, valine, acetate, glutamine, carnitine, inositol, β-glucose, tyrosine and for-

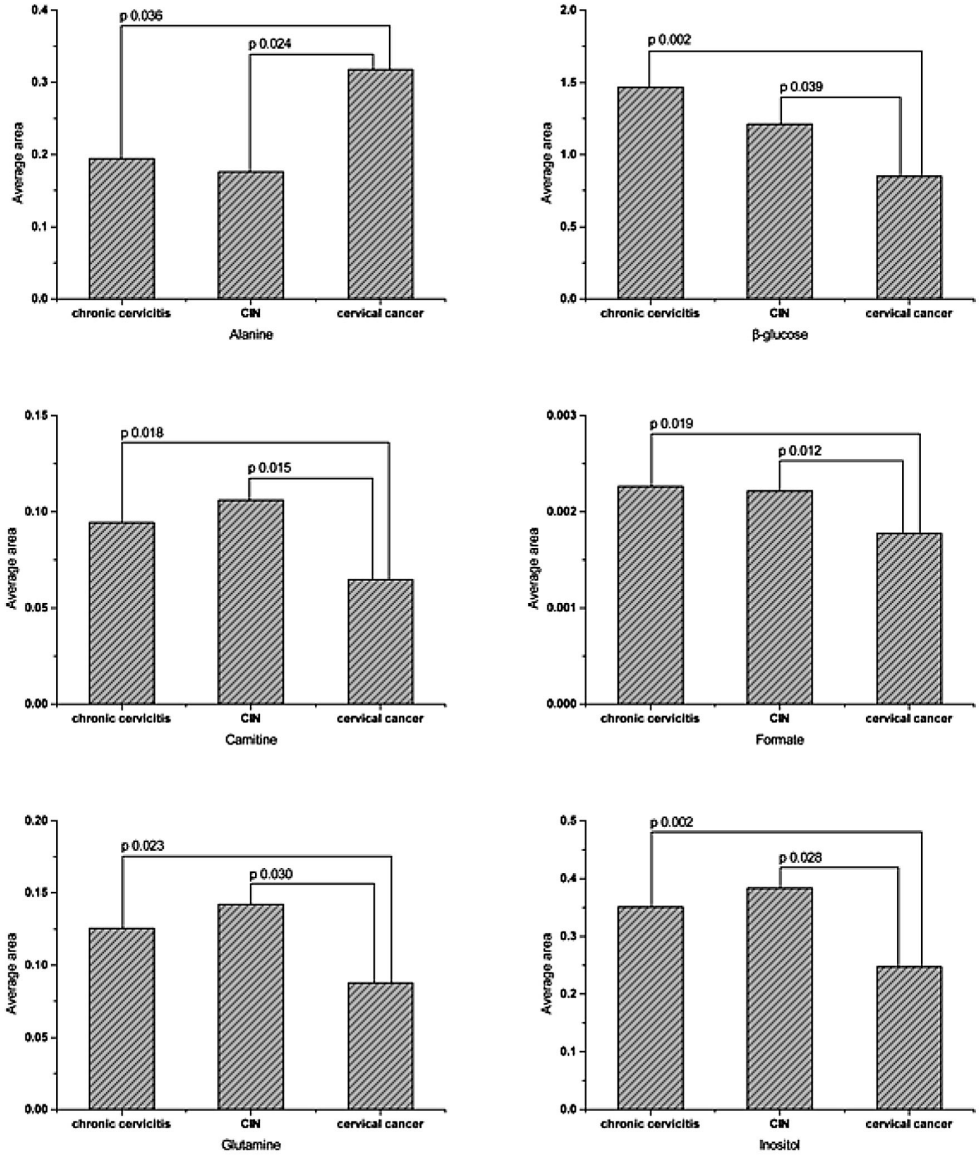

Figure 3. — A significant difference for the metabolites which have both $p<0.05$ between cervical cancer and its precancerous diseases, p values are marked in the figure.

mate, as well as higher abundance of very-low-density lipoprotein (VLDL), alanine, and glycine in their serum compared with the CIN. Compared with cervical cancer, there was only the abundance of acetate in a patient with chronic cervicitis, differing from the CIN, which had a higher level in cancer. Nevertheless, changes in the concentrations of serum metabolite with cervical cancer, and CIN patients clearly pointed to an altered energy metabolism. The relatively lower level of glucose was the same with cervical cancer tissue [30], in which the decreased glucose levels in tissue was due to elevated energy requirement. Potential precursors of glucose in gluconeogenesis, such as alanine and glycine, were found at higher levels in patients with cervical cancer. Levels of alanine and glycine in cervical cancer were different with oral cancer [24]; these may be caused by its hypoxia and ischemia. However, the increasing energy expenditure and metabolism may be followed by the development of these diseases, which may lead these precursors at higher concentrations and glucose as major energy source. These could be the reason of such changes of metabolites from CIN to cervical cancer. Furthermore, several intermediates of tricarboxylic acid (TCA) cycle were found at a lower concentration in patients with cervical cancer, such as glutamine, tyrosine, valine, and formate, which suggest a suppressed TCA cycle. Formate, as an intermediate of TCA cycle and pyrimidine and amino acid degradation, was less concentrated in patients with cervical cancer. As required by cells as an amino donor, glutamine was the synthetic precursor of α-ketoglutarate, tyrosine was precursor of fumarate, and valine and isoleucine were the precursor of succinate, which may be affected by TCA cycle. Furthermore, glutamine can

improve cell's antioxidant capacity by maintaining the reserve of glutathione. Low level of glutamine in cervical cancer may limit the synthesis of glutathione, which decreased antioxidant capacity. Isoleucine was also the synthetic precursor of acetyl-CoA, which was the raw material of TCA cycle. Due to the degradation of acetoacetyl-CoA that can produce tyrosine, its low concentration may also indicate a suppressed degradation of acetoacetyl-CoA, so can TCA cycle. These metabolites' abundance may represent an unclenched energy metabolism in cervical cancer patients and its precursors. These results suggested a suppressed TCA cycle for energy metabolism. In summary, the elevated energy requirement and the suppressed TCA cycle represented a typical signature that cancer may rely on glycolysis as its main energy source.

Urea cycle can use breakdown products of amino acid to feed into the TCA cycle. Based on this, the blood levels of most essential and non-essential amino acids, such as valine, isoleucine, glycine, tyrosine, and so on, were found to be lower in patients with cervical cancer. However, the reduction of amino acids may also be caused by cervical epithelial hyperplasia, accelerated tissue metabolism, increased protein synthesis, and reduced protein decomposition.

Due to the increased rate of lipid metabolism in response to the tissue injury caused by cancer, patients with cervical cancer may have increased level of VLDL, unsaturated lipid, choline, inositol, and acetone, since they were products of lipid metabolism. The role of these metabolites during the progression of cervical cancer had also been documented by previous works. According to literature [38], lipoprotein was closely related with cancer stage by studying the serum of 40 Lewis lung carcinoma patients, and the main function of VLDL was to transport endogenous triglycerides. Inositol had lower concentration in cervical caner, which played the same role as choline. Low level of inositol may indicate that the integrity of cell membrane was reduced in cancer patients. Compared with CIN and chronic cervicitis, carnitine had a low level in cervical cancer, which could enhance fat metabolism. These metabolites had described an increased cell turnover and represented the reversibility of cervical cancer. These reflect the lipolysis pathway could be a backup mechanism for energy production. The increased energy consumption, lipid mobilization, and transport may also be initiated in its precursors.

Conclusion

In this study, metabolomics analysis of cervical cancer, CIN, and chronic cervicitis were characterized by ^{1}H NMR, and analyzed by PLS-DA. The results showed that the sample points of these three female diseases had achieved discrimination. It indicated that 20 metabolites had contributed significance for the classification of diseases and 12 of them had statistical significance. Moreover, this method allowed distinct differentiations among the three diseases. Six metabolites, alanine, glutamine, carnitine, inositol, β-glucose, and formate, had significant differences between cervical cancer and its precancerous diseases, which may be identified as potential biomarkers and used for discrimination of cervical cancer.

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