Proteomic Screening for Serum Biomarkers for Cervical Cancer and Their Clinical Significance

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Background: The present study aimed to determine serum markers for cervical cancer (CC) and to provide valuable references for clinical diagnosis and treatment.

Material/Methods: Serum samples were collected from age-matched healthy control women, and from female CC patients before and after surgery. Serum biomarkers were selected by comparing serum peptides profiles among the 3 groups by magnetic bead-based weak cation – exchange chromatography fractionation combined with matrix-assisted laser desorption/ionization-time of flight mass spectrometry. Probable serum biomarkers for cervical cancer were then further identified by liquid chromatography-electrospray ionization-tandem mass spectrometry system and the identified proteins were verified by enzyme-linked immunosorbent assay (ELISA).

Results: Three peptide biomarkers were identified for distinguishing CC patients from normal individuals, and distinguishing preoperative CC patients from postoperative CC patients. Of these 3 identified protein peptide regions, 2 peptide regions – TKT (Peak 2, 2435.63 m/z, 499–524) and FGA (Peak 4, 2761.79 m/z, 603–629) – were identified as upregulated markers, and peptide region of APOA1 (Peak 9, 2575.3 m/z, 245–260) was identified as a downregulated biomarker in preoperative CC patients compared with healthy women.

Conclusions: The present study provides a new method for identifying potential serum biomarkers for CC patients.

MeSH Keywords: Biomarkers, Pharmacological • Proteomics • Uterine Cervical Neoplasms

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Background

Cervical cancer (CC) is the third most common cause of mortalities in females among all cancers, and seriously harms women's health [1]. The prognosis of early cervical cancer (stages I–II) is better; the 5-year survival rate is over 65%, and the recurrence rate after radical operation is 15–30%. The change in 5-year survival rate depends mainly on severity of the disease, and treatment for advanced cervical cancer (IIb–IV) is mainly combined with radiotherapy and chemotherapy. However, the prognosis is poor, and the 5-year survival rate of stage IV is less than 15% [2]. At present, the poor prognosis of cervical cancer is mainly due to the following factors: late International Federation of Gynecology and Obstetrics (FIGO) staging, large tumor volume, deep interstitial infiltration, vascular tumor thrombus, and lymph node metastasis. However, these clinicopathological features cannot predict prognosis very well [3]. Many methods, such as cytological examinations, tissue examinations, and imaging examinations, are used for the preoperative diagnosis of CC, but the accuracy of diagnosis with these methods is still low. Therefore, accurate and predictable early screening of CC has become urgent in clinical practice of CC. Tumor biomarkers in serum are of great significance in the treatment of CC patients. It is reported that rising serum CA 125 levels in pre-treated patients during follow-up may precede the clinical diagnosis of recurrent cervical adenocarcinoma [4]. Serum levels of vascular endothelial growth factor and C-reactive protein are often elevated in patients with CC, but decreased significantly after successful treatment, so they can be considered as additional independent prognostic parameters in patients with CC [5–7]. In addition, the ratio of serum Angiopoietin-1 to Angiopoietin-2 may also be a valuable diagnostic and prognostic biomarker for CC [8]. In addition, more prognostic markers for CC are also obtained by further research methods. According to bioinformatics study, 5 genes are found to be potential independent prognostic biomarkers for patients with CC [9]. G protein-coupled estrogen receptor (GPER) is detected in various subcellular staining patterns and serves as a prognostic marker at early stage of CC [10]. Utilization of novel biomarkers may facilitate personalized treatment and improve outcomes in the treatment for CC [11]. SEL1L, Notch3, SOCS3, c-Met, and Ki-67/MIB-1 also have potential values as diagnostic and prognostic indicators for CC [12–14]. Although studies have already shown diagnostic biomarkers for CC patients, there have been few reports on potential prognostic serum markers for CC after surgery.

The recurrence rate of patients with CC is high, and early diagnosis cannot determine recovery after surgical treatments. Therefore, discovery of prognostic markers for CC will improve clinical treatment efficacy. Proteomics approaches have been used to understand the correlation between HPV virus and CC pathology, and to discover putative biomarkers for early cervical cancer diagnosis and drug mode of action [15,16]. As a tool for biomarker searching, mass spectrometry has led to the discovery of easily available diagnostic tests in gynecological oncology, with emphasis on clinical proteomics [17]. In the technologies used to identify diagnostic markers at early stages of CC, significant advances have been achieved in 3 key areas: protein profiling, multidimensional liquid chromatography combined with cutting-edge mass spectrometry, and high-throughput validation techniques. These new technologies have significant promise in identifying more robust, sensitive, and specific markers for CC [18].

In this study, we aimed to discover serum markers with better prognosis of CC through serum proteomics analysis, and to provide a reliable reference for clinical surgical treatment.

Material and Methods

Patients

A total of 39 female CC patients before surgery and 28 female CC patients after surgery who received diagnosis and treatment at the Affiliated Hospital of Yan'an University between July 2014 and July 2016 were included in the present study (Table 1). In addition, 50 age-matched healthy subjects were included into the control group. The inclusion criteria were: i) patients who had complete medical records; ii) patients who had new primary cervical cancer; iii) patients who were clinically staged according to FIGO staging standard; iv) patients who had no other complications; v) patients who received radical hysterectomy + double appendage excision + pelvic lymph node dissection. The exclusion criteria were: i) patients who had chemotherapy alone or concurrent chemoradiotherapy; ii) patients who received surgical treatment in other hospitals; iii) patients who had incomplete diagnosis time and data. Serum samples were collected from healthy subjects and CC patients. Seven days after the operation, non-fasting blood samples (5 ml) were collected, anticoagulated with sodium citrate, and kept at room temperature for 30 min before being centrifuged at 3000 rpm and 4°C for 20 min. Serum samples were aliquoted into 100 μL in each tube and stored at -80°C until use to avoid repeated freezing and thawing. All procedures performed in the current study were approved by the Ethics Committee of Yan'an University. Written informed consent was obtained from all patients or their families.

Matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS)

Peptidome separation of all serum samples were performed using magnetic bead-based weak cation-exchange chromatography (MB-WCX) (ClinProt purification reagent sets; Bruker

Table 1. Cervical cancer patient characteristics (N=39).

Parameters		No. of cases
Age (year)	Median (range)	45
	≤45	19
	>45	20
FIGO stage	IA1–IA2	1
	IB1	16
	IB2–IIA2	12
	IIB	8
	IIIA–IV	2
Pathological type	Squamous carcinoma	29
	Adenocarcinoma	10
Differentiation	Well differentiated	10
	Moderately differentiated	16
	Poorly differentiated	13
Pelvic lymph node metastasis	Negative	20
	Positive	11
	Unknown	8
Tumor size	≤2 cm	26
	>2 cm	13
Lymphovascular space invasion	Negative	24
	Positive	15

Daltonics, Bremen, Germany) according to the manufacturer's protocol. Serum samples were added to MB-WCX beads, and samples were incubated at room temperature after thorough stirring. Then, the supernatant was removed. After washing away the impurities, the peptide fraction was eluted from magnetic beads into stabilization buffer. The eluted peptides (1 µl) were spotted onto the target and let dry, and then α-cyano-4-hydroxy-cinnamic acid (1 µL) in 50% acetonitrile and 0.5% trifluoroacetic acid mix was added to the surface. Targets were tested immediately by calibrated MALDI-TOF MS (Bruker Daltonics, Bremen, Germany), using FlexControl version 3.0 software in an optimized measuring protocol from mass range 1–10 kDa. All tests were performed in a blinded manner, including serum analysis of different groups. Data analysis was performed by Autoflex Analysis version 3.0 software (Bruker Daltonics, Bremen, Germany). Peptide patterns were analyzed by ClinProTools version 2.2 software (Bruker Daltonics, Bremen, Germany). A mass tolerance of 10 ppm for intact peptide masses and 0.6 Da for fragmented ions were permitted. Samples (50 µl) was mixed

with a mixture (800 µl) of 5% acetonitrile and 0.5% formic acid, and centrifuged at 7000×g and 20°C for 40 min before condensing to 500 µl. Then, the samples were desalinized and washed with 70% methanol and 0.5% formic acid before condensing to 100 µl. Then, the samples (20 µl) underwent one-dimensional ultra-high-performance liquid chromatography (Nano Aquity UPLC; Waters Corporation, Milford, USA) according to the manufacturer's manual. Then, gradient elution was performed.

Liquid chromatography-electrospray ionization-tandem mass spectrometry system (LC-ESI-MS/MS)

The selected peptide biomarkers were further separated by a nano-LC-ESI-MS/MS consisting of an ACQUITY UPLC system and an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a Nano-ESI source. Settings of the Nano Ion Source were as follows: spray voltage, 1.8 kV; MS scan time, 60 min; and scanning range from 400 to 2000 m/z. The first-order scan (MS) used Obitrap and the resolution was set to 100 000; the CID and the second-level scan used LTQ; the intensity was selected in the MS spectrum. A strong isotope of 10 ions was used as the parent ion for MS/MS (single-charge exclusion, not as parent ion). The identified sequence data were then subjected to a mascot database search using Bioworks Browser 3.3.1 SP1 (Bioworks, Victor, NY, USA) to match the corresponding full-length protein. A mass tolerance of 10 ppm for intact peptide masses and 0.6 Da for fragmented ions was permitted. The database was uniprot_human.fasta. The parent ion error was set to 100 ppm, the fragment ion error was set to 1 Da, the cleavage was non-enzymatic, and the modification was M (Methionine) methionine oxidation. The search result parameter was set to delta cn ≥0.10, 2 charges Xcorr 2.6, 3 charges Xcorr 3.1, and 3 charges Xcorr 3.5. The acquired data were searched against the UniProt protein sequence database of HUMAN (http://www.uniprot.org).

ELISA

The levels of TKT, APOA1 and FGA were determined using human ELISA Kits for TKT (DRE13075), APOA1 (DRE10228), and FGA (DRE129395130) (Shanghai Haring Biotechnology Co. Ltd., Shanghai, China). All samples were diluted at a ratio of 1: 5 and analyzed in duplicate ELISA wells, according to the respective ELISA kit protocols. The absorbance of individual samples and ELISA test kits standards were measured at the wavelength specified by each test kit, using a FLUOstar Omega reader (BMG LABTECH GmbH, Germany). Standard curves were generated to determine the concentrations of TKT, APOA1, and FGA.

Statistical analysis

The results were analyzed using GraphPad Prism version 5.0 (GraphPad Software, La Jolla, CA, USA). The data are expressed
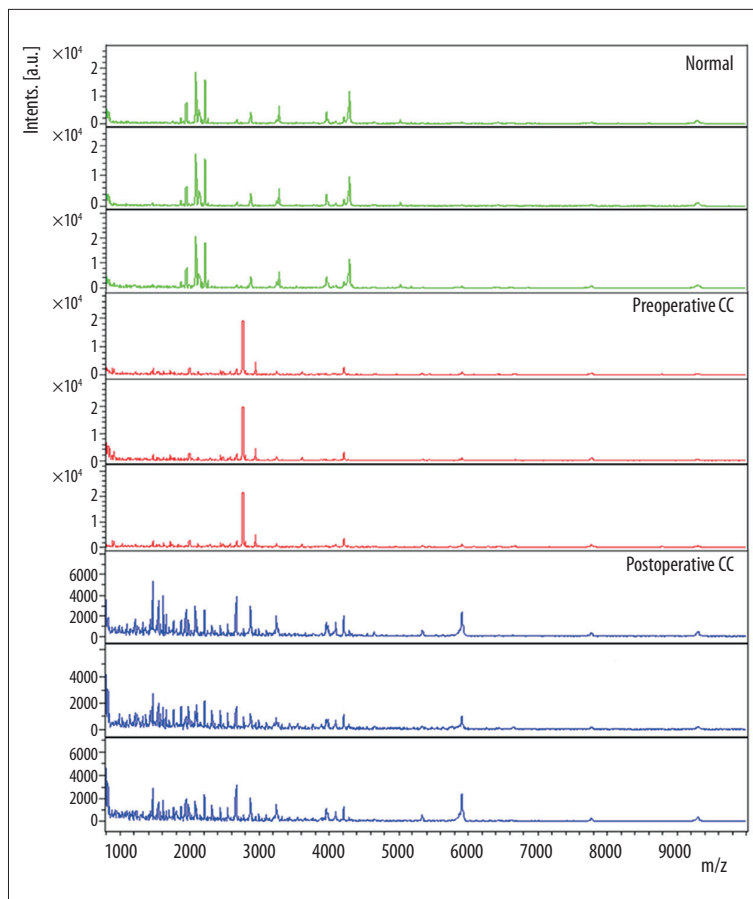
Figure 1. Reproducibility of mass spectra generated in individuals from different groups. Representative mass spectra of a normal control (green), a preoperative (red) CC patient, and a corresponding postoperative (blue) CC patient. Mass range, 1 to 10 kDa.

as means ± standard deviations. Data were tested for normality. Multigroup measurement data were analyzed using one-way ANOVA. In case of homogeneity of variance, least significant difference and Student-Newman-Keuls methods were used; in the case of variance heterogeneity, the non-parametric Friedman test was adopted. Comparison between 2 groups was carried out using the *t* test. P<0.05 indicated statistically significant differences.

Results

Expression of 3 peptides after operation is lower than that before operation

For serum proteomic profiling, triplicate detection was carried out. MB-WCX fractionation and MALDI-TOF MS polypeptide fingerprint profiles from 1 to 10 kDa showed that mass spectra differed among normal controls (green), preoperative (red), and postoperative (blue) CC tissues (Figure 1). ClinProTools analysis of all groups in this mass range showed up to 151 peaks, and the expression of 9 of the peaks were significantly different among normal controls and preoperative and postoperative CC samples (P<0.0001, Intents fold

changes >1.5 or <0.6). Peaks 2,4,7,9 were upregulated in preoperative CC patients compared with normal controls. Peaks 1–4, 8, 9 were downregulated in postoperative patients compared with preoperative patients. Three peptide peaks (Peak 2, m/z: 2435.63; Peak 4, m/z: 2761.79; Peak 9, m/z: 2575.3) showed significant values when comparing preoperative patients and postoperative patients to normal controls, indicating that they could be candidate prognostic serum biomarkers for CC (Table 2). In component analysis, a bivariate plot of normal controls (green), preoperative (red) and postoperative (blue) CC patients showed few overlapping regions (Figure 2A). Comparison of the spectra of these 3 peaks (m/z: 2435.63, 2761.79, 2575.3 Da) in all groups and their mean expression levels is shown in Figure 2B. Peptide mass spectra comparisons of the 3 peaks in all samples (Figure 2C–2E) were consistent with the numerical results in Table 2. These results suggest that expression of 3 peptides after operation was lower than that before the operation.

The 3 peaks (m/z: 2435.63, 2575.3, 2761.79 Da) may be prognostic serum biomarkers for CC

To compare the profiles of all samples about the 3 peaks (m/z: 2435.63, 2575.3, 2761.79 Da) between preoperative CC

Table 2. Mean levels of 9 differentially expressed peaks in healthy controls and in preoperative and postoperative CC patients.

Peaks	Mass (Da)	P value	Normal	Preoperative	Postoperative	Fold (preoperative/ normal)	Fold (postoperative/ preoperative)
1	1998.48	<0.000001	4.74±2.65	5.55±2.55	2.63±0.87	1.17	0.50
2	2435.63	<0.000001	2.77±1.16	4.52±2.33	2.26±0.74	1.63	0.51
3	1722.36	<0.000001	3.43±2.05	5.05±4.62	2.35±0.6	1.47	0.46
4	2761.79	<0.000001	17.09±15.32	39.02±29.21	14.62±10.04	2.28	0.37
5	879.99	<0.000001	5.72±1.54	7.29±2.36	5.03±2.51	1.27	0.69
6	1470.31	<0.000001	4.84±1.53	6.6±2.72	4.73±2.48	1.36	0.72
7	1949.62	<0.000001	7.7±5.06	11.28±9.56	9.43±6.91	2.73	0.84
8	826.61	<0.000001	14.86±3.35	19.72±11.22	9.87±6.72	1.31	0.51
9	2575.3	<0.000001	3.38±0.96	4.06±2.41	2.23±0.91	1.50	0.54

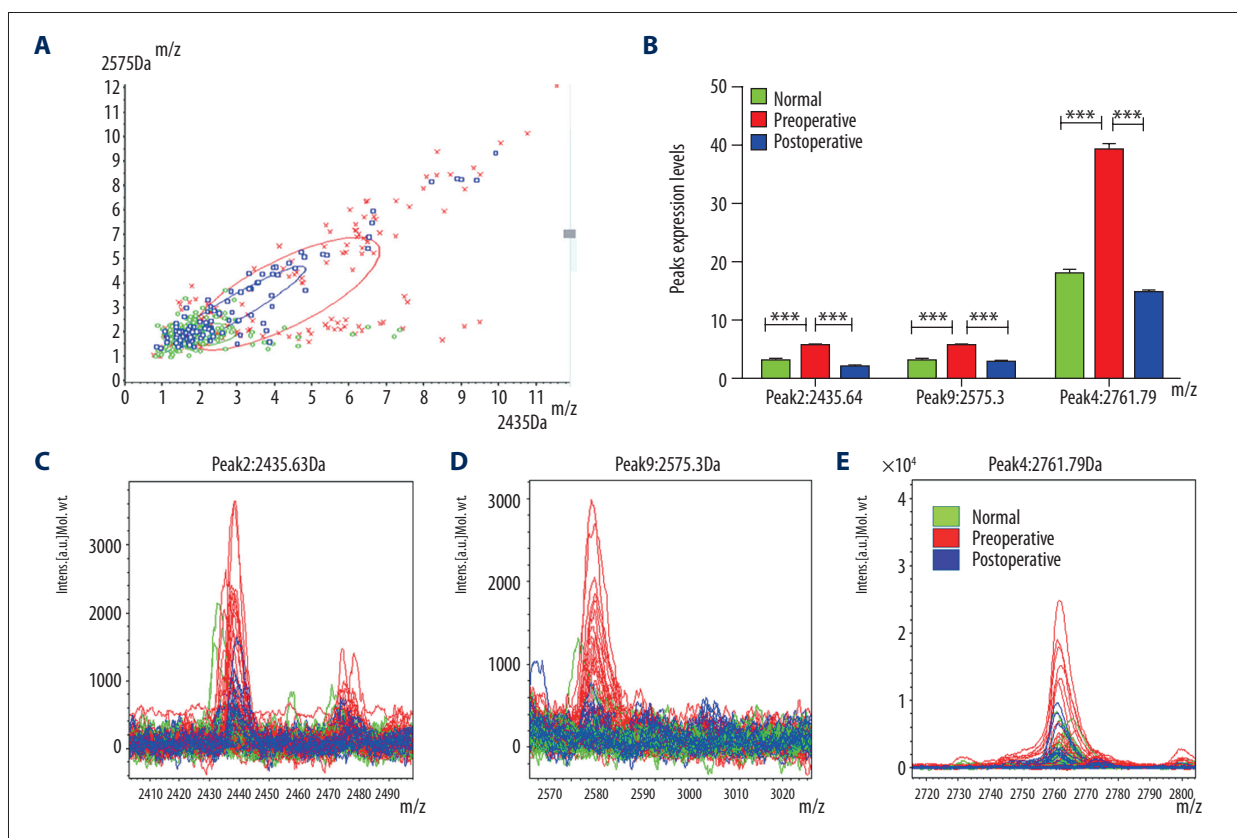Figure 2. Serum proteomic profiling analysis of normal subjects, preoperative CC patients, and postoperative CC patients. (A) Bivariate plot of normal controls (green), preoperative CC patients (red), and postoperative CC patients (blue) in component analysis with the 2 most differentiated peaks (m/z: 2435.63, 2575.3 Da). Each dot represents a sample, and different colors represent different groups. X and Y axes show sample distribution using 2 peptides with the most difference at 2-dimensional levels. (B) Average expression levels of 3 potential biomarkers in normal controls (green), preoperative CC patients (red), and postoperative CC patients (blue). Values are expressed as means ±SD. (C–E) Spectra comparison of the 3 potential biomarkers (m/z: 2435.63, 2761.79, 2575.3 Da) in normal controls (green) and in preoperative CC patients (red) and postoperative CC patients (blue).
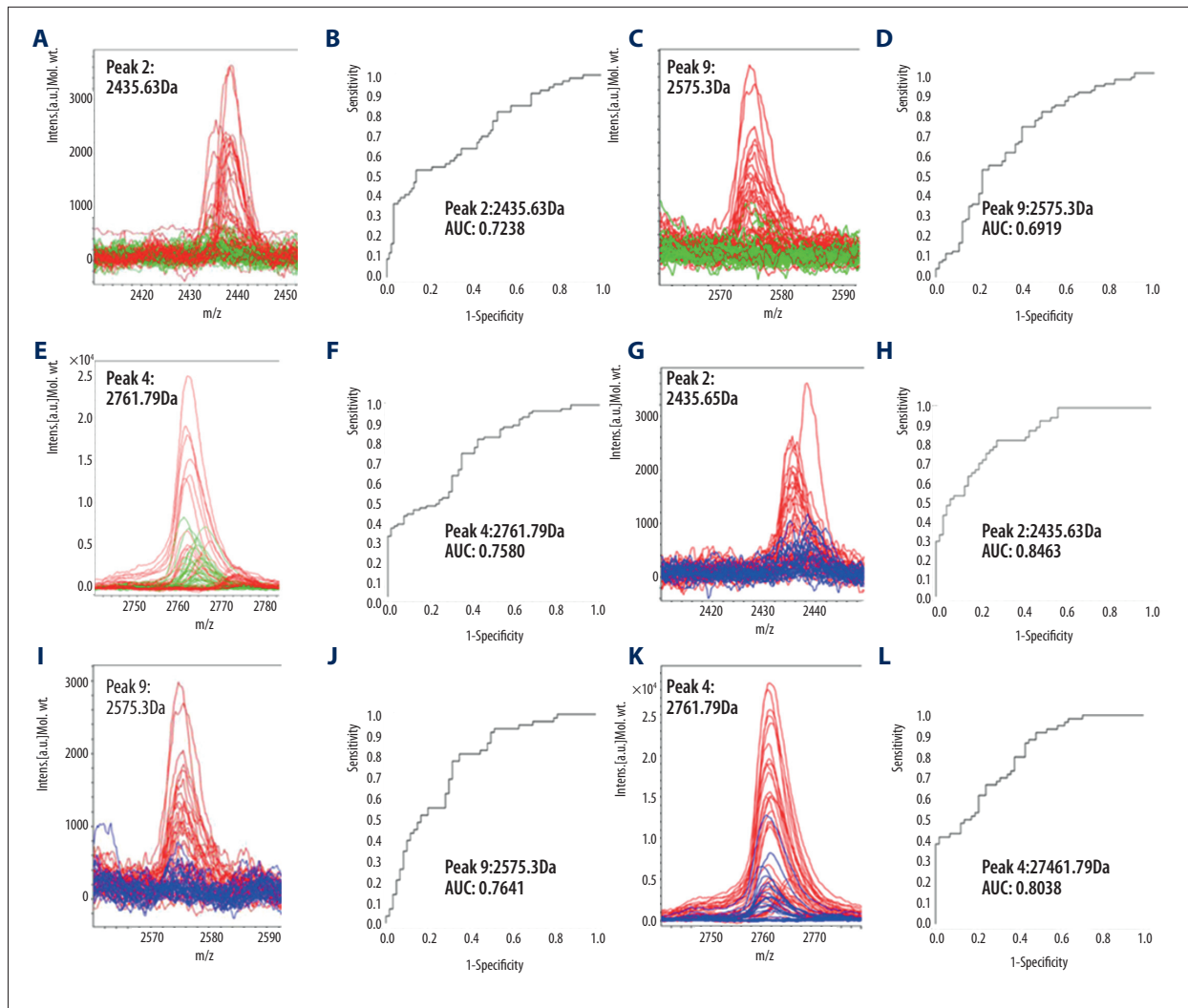
Figure 3. Comparative analysis of potential biomarkers in different groups. (**A, C, E**) Respective comparison of spectra of the 3 peaks between preoperative CC patients and normal controls. (**B, D, F**) ROC curves for the corresponding differentially expressed peaks with AUC values. (**G, I, K**) Respective comparison of spectra of the 3 peaks between preoperative CC patients and postoperative CC patients. (**H, J, L**) ROC curves for the corresponding differentially expressed peaks with AUC values.

patients (red) and healthy controls (green), receiving operating characteristic (ROC) curves were plotted (Figure 3A, 3C, 3E). The area under the curve (AUC) values of these 3 peaks were 0.7238 (Peak 2, m/z: 2435.63), 0.6919 (Peak 9, m/z: 2575.3), and 0.7580 (Peak 4, m/z: 2761.79) (Figure 3B, 3D, 3F). All profiles of the peaks showed a tendency close to normal levels after the operation (Figure 3G, 3I, 3K), and the AUC values of the 3 peaks between postoperative CC patients (blue) and normal controls (green) were 0.8463 (Peak 2, m/z: 2435.63), 0.7641 (Peak 9, m/z: 2575.3), and 0.8038 (Peak 4, m/z: 2761.79) (Figure 3H, 3J, 3L). The results indicate that the 3 peaks may be prognostic serum biomarkers for CC.

TKT and FGA are serum markers for surgical prognosis of CC patients

To identify and confirm the sequences of the 3 peptide peaks, LC-ESI-MS/MS and Uniprot database were used. MS/MS fragmentations of the 3 peaks were identified as the following peptide sequences: K.DDQVTVIGAGVTLHEALAAAELLK.K (2435.63 m/z); V.LESFKVSFLSALEEYTKKLNTQ.- (2575.3 m/z); K.MADEAGSEADHEGTHSTKRGHAKSRP.V (2761.79 m/z) (Table 3). They were further identified as regions of Transketolase (2435.63 m/z, 499-524, TKT), Apolipoprotein A-I precursor (peak 2575.3 m/z, 245-260, APOA1), and Isoform 1 of Fibrinogen alpha chain precursor (peak 2761.79 m/z, 603–629, FGA) (Figure 4A–4C). To determine the screened serum peptide biomarkers, serum concentrations of TKT, APOA1 and FGA were

Table 3. Sequence Identification of the 3 differentially expressed peaks in healthy controls and in preoperative and postoperative CC patients.

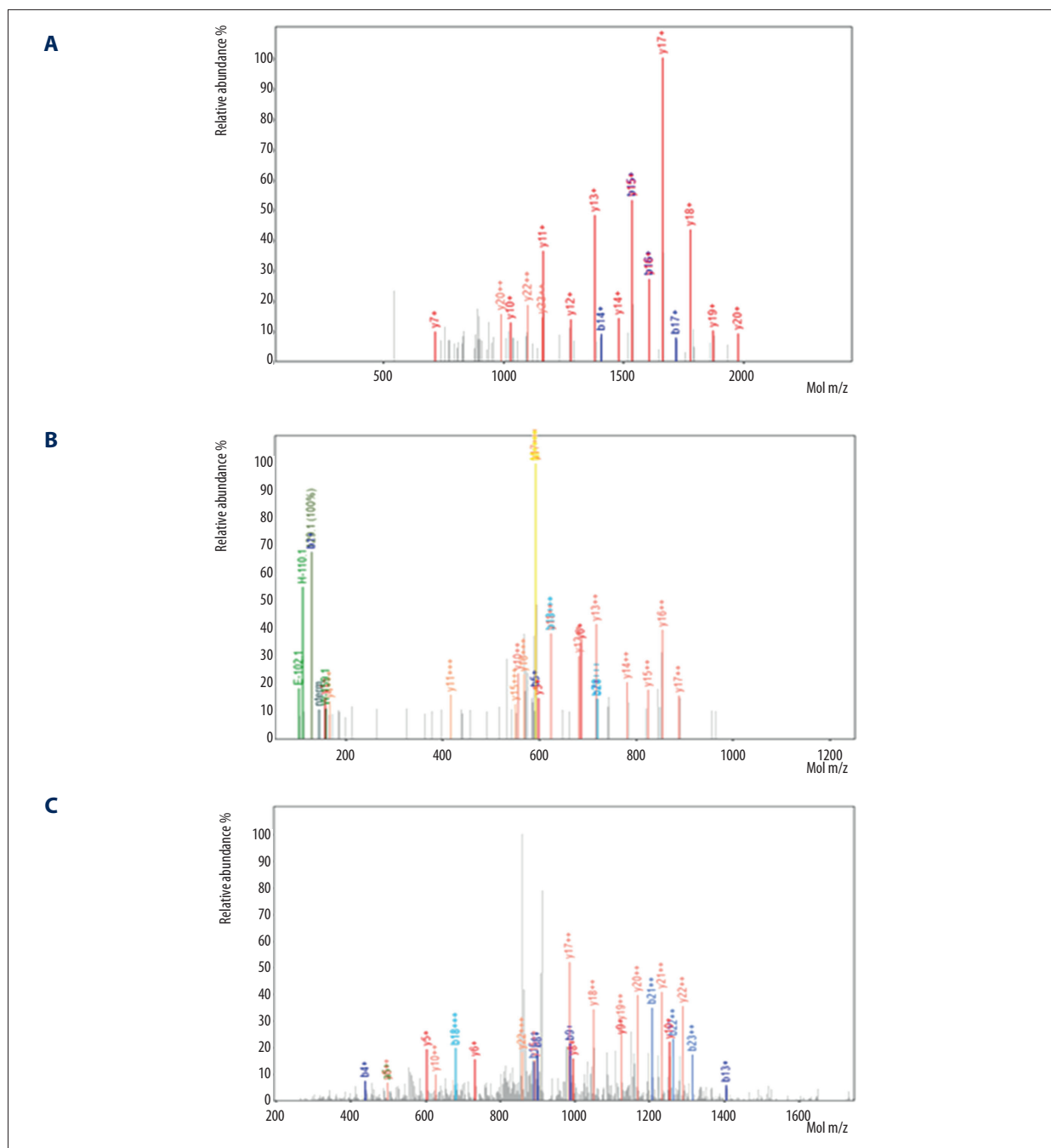| Mass (Da)	Identity	Peptide regions	Uniprot ID	Peptide sequence
2435.63	TKT	499–524	P29401TKT_HUMAN	K.DDQVTVIGAGVTLHEALAAAELLK.K
2575.3	APOA1	245–260	P02647APOA1_HUMAN	V.LESFKVSFLSALEEYTKKLNTQ.-
2761.79	FGA	603–629	P02671FIBA-HUMAN	K.MADEAGSEADHEGTHSTKRGHAKSRP.V

Figure 4. LC-ESI-MS/MS spectra of peptides with m/z of 2435.63, 2575.3, and 2761.79 Da, identified as serum peptides of (**A**) TKT, (**B**) APOA1, and (**C**) FGA.
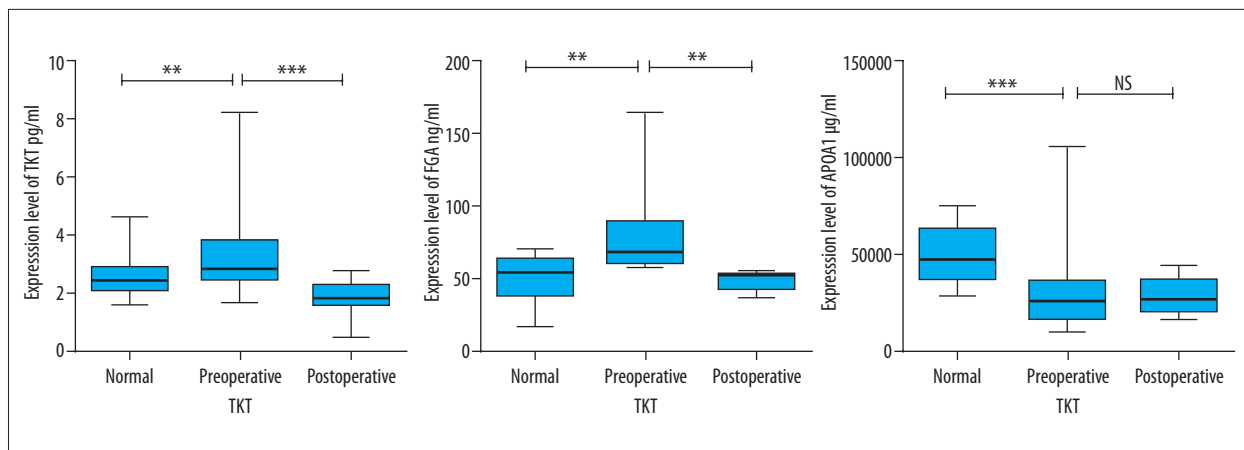
Figure 5. Concentration of the identified peptides (TKT, APOA1, and FGA) in healthy controls and in preoperative and postoperative CC patients. Statistical analysis was performed using Prism 5.0 software. All data are represented as means ±SD.

examined by ELISA from normal controls, preoperative and postoperative CC samples. The concentration of TKT in preoperative CC samples (3.58±0.40) was significantly higher than normal controls (2.62±0.39) (P<0.05), and that in postoperative CC tissues (1.91±0.27) was significantly lower than that in preoperative CC tissues (P<0.05). Concentration of APOA1 in preoperative CC patients (32.98±14.88) was significantly lower than that of normal controls (54.93±26.26) (P<0.05), but no difference was observed between preoperative tissues and postoperative CC patients (28.11±8.27) (P>0.05). Concentration of FGA in preoperative CC tissues (398.99±157.73) was significantly higher than that of normal controls (268.81±68.22) (P<0.05), and that in postoperative CC tissues (245.06±23.68) was significantly lower than that in preoperative CC tissues (P<0.05) (Figure 5). Of note, expression of TKT and FGA (m/z: 2435.63, 2761.79 Da) in preoperative CC tissues was higher than that in normal controls (P<0.05), but that in postoperative CC tissues was close to that in normal controls (P>0.05). A similar tendency was also observed in peptides analysis (Figure 5). Expression of APOA1 (m/z: 2575.3 Da) in the preoperative CC group was significantly lower than in normal controls (P<0.05), but its expression in postoperative CC tissues did not reach to a level close to that of normal controls after the operation (P>0.05). These results indicate that TKT and FGA are serum markers for surgical prognosis of CC patients.

Discussion

TKT, a crucial enzyme in the nonoxidative pathway of the pentose phosphate pathway (PPP), is required for cancer growth. Cancer cells experience an increase in oxidative stress, and PPP is a major biochemical pathway involved. It is reported that antioxidants are beneficial to cancer growth, and TKT targeting pathways generate antioxidants [19]. Some studies showed that high TKT expression level plays an important role in the

development of cancer, including CC [20–23]. Consistently, the data in the present study demonstrate that TKT is upregulated in preoperative CC patients compared with normal controls. TKTL1 plays an important role in total TKT activity and CC cell proliferation in the nonoxidative pathway of PPP [23]. The deregulated miR-497/TKT axis implies that PPP is beneficial to chemoresistance, and targeting TKT may have therapeutic benefits in the treatment for CC [22]. Identification of PHLDA2, TKT, and P4HA2 genes associated with patient-derived xenograft engraftment also provides a novel prognostic biomarker and therapeutic targets in triple-negative breast cancer [24]. These findings are consistent with our finding that TKT expression after treatment is close to that in normal subjects, indicating that TKT may be a prognostic marker for CC.

FGA is human fibrinogen that is synthesized in the liver and formed by 2 symmetrical molecules accompanied by 3 different polypeptide chains [25], and it is a marker for various tumors [26–28]. It is reported that FGA is associated with endometriosis pathogenesis [29]. The FGA peptide regions identified in the present study are potential biomarkers for preoperative CC, and respond well to treatment. Hyperfibrinogenemia and thrombocytosis usually occur in cancer patients, and contribute to cancer cell growth, progression, and metastasis. Similar to the results of the present study, a previous study showed that FGA is a valuable biomarker for predicting recurrence in patients with early-stage CC [30].

APOA1 participates in the reverse transport of cholesterol from tissues to the liver for excretion by promoting cholesterol efflux from tissues and by acting as a cofactor for lecithin cholesterol acyltransferase (LCAT). Lipid metabolism-related proteins (APOA4, APOA1, APOE) are dysfunctional in cervical squamous cell carcinoma, and identified as biomarker signatures for CC [31]. APOA1 is a major protein component of HDL, and it is suggested that the ratio of HDL-C to APOA1

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may be a risk marker not just for cardiovascular disease, but also for cancer mortality [32]. Similarly, APOA1 is also a biomarker for endometrial cancer and cervical high-grade squamous intraepithelial lesions [33,34]. The present study for the first time determined that APOA1 is a candidate prognostic serum marker for CC.

However, our study still has some limitation. For example, obvious heterogeneity exists among each patient group, including age, stage, and tumor type, which collectively reduces the power of marker identification.

Conclusions

In conclusion, the present study reveals 3 prognostic serum biomarkers for CC patients. ClinProTools software analysis of serum peptidome by MB-WCX fractionation combined with MALDI-TOF MS generated differentiated expression peaks among preoperative and postoperative CC patients and normal controls. Three identified peptides – TKT (2435.63 m/z, 499–524), APOA1 (peak 2575.3 m/z, 245–260), and FGA (peak 2761.79 m/z, 603–629) – are considered to be prognostic serum biomarkers for CC patients. The present study also provides a valuable new approach for the identification of prognostic or predictive markers for recurrent CC, potentially leading to the design of novel diagnostic and therapeutic strategies. The limitation of the present study is the small number of clinical samples. Further studies need to be performed to develop antibodies against these identified candidate markers.

Conflicts of interest

None.

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