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RESEARCH ARTICLE

The association of a distinct plasma proteomic profile with the cervical high-grade squamous intraepithelial lesion of Uyghur women: a 2D liquid-phase chromatography/mass spectrometry study

Xia Guo [1], Guzalnur Abliz[2], Halidan Reyimu[1], Feng Zhao[2], Nafeisha Kadeer[1], Rukeya Matsidik[1], Guizhen Wu[1], and Abulizi Abudula[1]

[1]*Xinjiang Key Laboratory of Molecular Biology and Endemic Diseases, Xinjiang Medical University, Urumqi, PR China and* [2]*The Affiliated Tumor Hospital of Xinjiang Medical University, Urumqi, PR China*

Abstract

Objective: To identify plasma protein biomarkers of cervical high-grade squamous intraepithelial lesion (HSIL) of Uyghur women by proteomics approach.

Methods: Plasma protein samples of Uyghur women with HSIL and chronic cervicitis were analyzed with 2D HPLC followed by detection of target proteins with Linear Trap Quadrupole Mass Spectrometer (LTQ MS/MS).

Results: We detected three upregulated and one downregulated protein peaks representing protein constituents distinguishing HSIL from controls by 2D HPLC, identified 31 target proteins by LTQ MS/MS. Further confirmed analysis with online software IPA® 8.7 and ELISA assay showed APOA1 and mTOR as potential biomarkers.

Conclusions: A distinct plasma proteomic profile may be associated with HSIL of Uyghur women.

Keywords: Low abundant plasma proteome, Uyghur women, high-grade squamous intraepithelial lesion, biomarkers, 2D liquid-phase chromatography

Introduction

Cervical cancer is the second morbidity malignant tumor of women worldwide which ranks just behind breast cancer. It takes of nearly 27 million women's lives every year and has been severely threatening the health and life of women (Yang et al. 2004; Cohen 2005; Ferlay et al. 2007). The prevalence rate of cervical cancer among Uyghur women in south Xinjiang (590/10 million) is four times of China's women's average prevalence (138/10 million); therefore, cervical cancer was listed as one of high-incidence endemic diseases in Xinjiang(Guzalnur et al. 2004; Suzuke et al. 2006). Patients admitted to hospital are more dominated by the late stage, losing the best time for treatment.

Epidemiology and cytology research evidence of cervical cancer showing that the developing process of cervical cancer, from dysplasia to invasive cervical cancer (ICC), is a continuous histological event. The Bethesda System (TBS 2001) for cervical cytology reporting classifies squamous intraepithelial lesions (SIL) into two categories: low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intraepithelial lesions (HSIL). HSIL referred to moderate to severe stage cervical intraepithelial neoplasia (CIN II and III) is the one well-documented precursor to ICC (Siddiqui 2000). HSIL persists, with approximately 40%–100% cases progressing to carcinoma, whereas most cases of LSIL may regress spontaneously (Correa 1988). Therefore, HSIL is usually treated with the surgery of loop electrocautery excision procedure (LEEP) or cervical conization, whereas LSIL is followed closely by Pap smear and/or biopsy (Ince et al. 2011).

Address for Correspondence: Prof. Abulizi Abudula, Xinjiang Key Laboratory of Molecular Biology and Endemic Diseases Xinjiang Medical University, Urumqi 830011, Xinjiang, PR China. E-mail: abulizi_de@126.com

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Histology from colposcopic-directed biopsies is considered the gold standard for the diagnosis of cervical dysplasia. Lugol's staining is used in cervical colposcopy to differentiate cervical HSIL from LSIL. However, it is well acknowledged issue, the so-called cytohistologic discrepancy that a certain number of women with HSIL histology are preceded by minimal cytological abnormality (Zuna et al. 2002; Massad et al. 2003; Gupta & Sodhani 2004), and may be resulted in delayed diagnosis and treatment. But the most determinative reason for this outcome lies on the lack of valuable objective indicators for the differentiate of cervical chronic inflammation, reactive hyperplasia and benign or malignant SIL.

Serum or plasma is the most accessible and minimally invasive, and routine sample in clinical practice, reflecting its communication with all cells, tissues and organs (William 2007; Cho & Cheng 2007; Linkov et al. 2007). It is essential to find key protein biomarkers associated plasma/serum that can be used to classify lesions with respect to organ of benign, inflammatory versus aggressive behavior. This may serve as an early diagnostic tool aiding for disease mechanism elucidation and determination of treatment strategy (Sullivan et al. 2001; Moore et al. 2008). In this study, we focused on establishing differential lower-abundant protein expression profile and identifying serological candidate biomarkers specifically for HSIL and benign gynecologic disease cervicitis as normal controls in Uyghur women using 2D liquid-phase chromatography in conjunction with nano-LC-MS/MS. Bioinformatic analysis within the framework of IPA® of candidate proteins was conducted to characterize the interacting proteins and network involved in putative linkages to cancer-relevant pathways. Thus, it is expected that the comprehensive analysis of the plasma proteome will play an important role in monitoring subtle changes in Uygur women with HSIL in order to realize precancerous blood-based tests as a routine health checkup.

Materials and methods

Materials

The PF 2D chemistry kit, purchased from Beckman Coulter (Fullerton, CA), contains the (high-performance chromatofocusing, HPCF) 1D column (250 × 2.1 mm), the (high-performance reversed phase, HPRP) 2D reversed-phase column (4.6 × 33 mm) containing 1.5 mm ODS1 nonporous silica beads (Eprogen, Darien, IL, USA). Two buffers, a start buffer (SB) and an elution buffer (EB), were used to generate the pH gradient on the column. The BCA protein assay kit was purchased from Novagen (Merck Biosciences, Germany). The ProteoMiner Kit was purchased from Bio-Rad (Hercules, CA). HPLC-grade water and acetonitrile were purchased from Merck (Whitehouse Station, NJ, USA). Trifluoroacetic acid was purchased from Sigma-Aldrich (St. Louis, MO, USA); sequencing grade trypsin was obtained from Promega (Madison, WI, USA). All other chemical reagents were obtained from Sigma.

Table 1. Clinical information on Uygur women specimens.

Sample name	Quantity	Mean age	FIGO stage
Chronic cervicitis	22	40 ± 1.67	
HSIL	21	50 ± 1.56	CIN2/3

HSIL, high-grade squamous intraepithelial lesion.

Uygur women plasma samples

All plasma samples (Table 1) were collected by the Department of Gynecology of the Affiliated Cancer Hospital in Xinjiang Medical University after ethic committee approval. Signed informed consent forms were obtained from all patients and all data were analyzed anonymously throughout the study. Human blood was obtained by venipuncture from each donor into evacuated blood collection tubes that contained anticoagulant. The diagnosis was confirmed as HSIL histologically in all cases. None of the patients received any treatment before the primary treatment described here. As normal controls, plasma samples were obtained from patients with benign gynecologic disease chronic cervicitis. All patients provided informed consent before collection of the plasma samples.

Depletion of the highly abundant plasma proteins by The ProteoMiner™ Protein Enrichment Kit

At first, 1 mL deionized water and 1 mL wash buffer were used to prepare column containing 100 µL settled beads and ready for sample binding. Then 1 mL of sample was add to the column and incubated by rotating for 2 h, followed by washing the column again with 1 mL wash buffer for several times. Then, 100 µL rehydrated elution reagent was added to the column followed by collection of eluent containing so-called low abundant plasma proteins.

Instrumentation

2D liquid-chromatography separations were carried out on the ProteomeLab™ PF 2D system controlled by the 32 Karat™ software from Beckman Coulter (Fullerton, CA), which contains two additional software packages for graphic representation of chromatography data called ProteoVue and DeltaVue. Samples were collected in a FC 204 fraction collector (Gilson) equipped with a platform to hold a total of eight 96-well deep-well microtiter plates. Operation of the procedure and methods were according to our previous research (GUO et al. 2009). Mass Spectrometry was performed on LTQ-Orbitrap mass spectrometer (Thermo Scientific) equipped with an Eksigent nano-LC system. Bioinformatic analysis was used by the software package of Ingenuity Pathway Analysis (Ingenuity Systems IPA®, Mountain View, CA, version 8.7).

Chromatofocusing HPLC (HPCF) – the first dimension

In order to ensure that the same quantity of every group samples were injected and differences were comparable. Therefore, prior to sample injection into the 1 mL sample loop, plasma protein concentrations were determined with

the BCA method and adjusted to a concentration of 1.0 mg/mL with first-dimension SB. First-dimension chromatofocusing was carried out at ambient room temperature at a flow rate of 0.2 mL/min. Before each run, the column was equilibrated with SB (pH 8.5) for about 120 min until a stable baseline value of pH 8.5 was achieved. Improved separation reproducibility was observed when the entire sample loop was filled with sample. Therefore, 1 mL of the adjusted plasma sample was routinely injected onto the first-dimension chromatofocusing column. Once the pH in the column achieved a stable pH at 8.5, the linear gradient of EB to pH of 4 was switched. The proteins remaining on the column at pH 4 were washed out by 1 M NaCl solution. UV detection was performed at 280 nm and the pH of the effluent was monitored using a flow through online pH probe. Fractions were collected into deep-well-plates at 0.3 pH intervals and stored at 4°C in the auto-sampler prior to injection into the reversed-phase column.

Reverse phase HPLC (HPRP) – the second dimension

The pI fractions were further separated on HPRP column packed with nonporous silica beads. Solvent A was 0.1% (v/v) trifluoroacetic acid (TFA) in water and solvent B was 0.08% (v/v) TFA in acetonitrile. The separation was done at 50°C at a flow rate of 0.75 mL/min. The gradient was run from 0% to 100% B in 35 min, followed by 100% B for 5 min and 100% A for 10 min. UV absorptions were monitored at 214 nm. The fractions were collected in 1 min time intervals into 96-well plates using the fraction collector Gilson FC 204 (Immunotech, a.s., Prague, Czech Republic) and stored at –40°C for further use.

2D protein map evaluation

2D plasma protein expression maps of two group samples (chronic cervicitis group and HSIL group) displaying protein isoelectric point (pI) versus protein hydrophobicity were generated by ProteoVue software (Beckmann Coulters, Fullerton CA). The intersample comparison was performed using DeltaVue software (Beckmann Coulters, Fullerton CA). ProteoVue software converts the UV peak intensity in the chromatograms from the second-dimension HPRP column of each pI fraction to a band and line format. The DeltaVue software allows side-by-side viewing of the second-dimension runs for two groups of samples so that differences in protein expression between them can be compared. This software quantitatively displays one protein map in shades of red and the other map in shades of green. The difference between the two maps is obtained by point-by-point subtraction or by area difference and displayed as a third map in the middle. The color (red or green) at a particular location in the difference map indicates which protein is more abundant, and the color brightness indicates the quantitative difference. The program also provides a means to obtain a quantitative number between the expression levels of protein in the two samples. The proteins displaying at least 2-fold change (Table 2) were selected for collection and analysis by mass spectrometry.

Trypsin digestion of plasma protein fractionations

In-solution digestion was performed with lyophilized aliquots from the reversed-phase (second dimension) fractionation step. Proteins in individual fractions were resuspended in 25 µL 8 M urea containing 50 mM ammonium bicarbonate pH 8 and then added to 100 mM dithiothreitol (DTT). 500 mM iodoacetamide (IAA) was then added to alkylate the proteins followed by 5 µL of DTT to stop the alkylation process. For each of these steps, 2 h incubation time was used. The samples were digested overnight with 2 µg of modified trypsin (Promega) at 37°C. After digestion, the samples were purified and concentrated using a C18 ZipTip (Millipore, Oslo, Norway) procedure. The organic phase was then evaporated off using vacuum-centrifugation and, to the residual solution, 20 µL 2% acetonitrile and 0.1% formic acid in MilliQ water was added prior to the LC-MS/MS analysis.

Table 2. Comparative and differential protein expression analysis of patients with HSIL and chronic cervicitis.

Peak	Lane	RT (L)	Data (L)	L/R	RT (R)	Data (R)	R/L	pH range
1	1	31.994	0.257	**–5.01***	31.994	–0.051	–0.2	8.60–8.52
2	2	29.767	1.006	**–6.776***	29.701	–0.148	–0.148	8.52–8.18
3	3	30.454	0.166	–1.427	30.387	–0.116	–0.701	8.18–7.88
4	5	32.111	0.561	**–4.112***	32.111	–0.136	–0.243	8.20–8.04
5	6	33.161	0.147	–0.989	33.144	–0.149	–1.011	8.04–7.74
6	23	20.927	0.063	–1.016	20.86	–0.062	–0.984	6.26–6.13
7	24	20.81	0.002	–0.087	20.744	–0.022	**–11.529***	5.58–5.27
8	28	31.421	–0.061	–0.081	31.421	0.753	–12.4*	3.92–N/A
9	29	32.111	–0.068	–0.061	32.111	1.112	–16.311*	N/A
10	30	33.027	–0.079	–0.214	33.094	0.369	–4.682*	N/A
11	33	36.587	–0.095	–0.18	36.587	0.525	–5.545*	N/A
12	34	18.63	1.436	–433.32*	18.63	–0.003	–0.002	N/A
13	42	23.997	0.081	–51.609*	24.017	–0.002	–0.019	N/A

Peak: number of different protein content; RT: time for the elution (min); Data: absorbance value of plasma protein at 214 nm; L/R: plasma protein absorption ratio of HSIL compared to cervicitis; R/L: plasma protein absorption ratio of cervicitis compared to HSIL. Asterisks in table represent the difference between two groups was more than twice. The peaks shown in bold are in the detection range from pH 8.5–4.0.

Nano-LC–LTQ-MS/MS analysis

Peptides from the trypsin-digested 2D HPLC fractions are eluted into an LTQ-Orbitrap mass spectrometer (Thermo Scientific) using an Eksigent nano-LC system equipped with a 75 μm id. × 15 cm PepMap 300, 5 μm, 300 Å, C18 analytical column (Dionex Berlin, Germany). The separation was done using gradient at 5% buffer B (buffer A: 2% acetonitrile and 0.1% formic acid in MilliQ water; buffer B: 80% ACN + 0.1% formic acid in MilliQ water) to 50–95% buffer B gradient over 130 min using 300 nL/min flow. The electrospray voltage was set to 2.2 kV. The mass spectrometer was used in positive mode. Full scans were performed in the Orbitrap using the m/z range from 350 to 1600 Da. Collision induced dissociation (CID) was used with a normalized collision energy of 35% and capillary temperature was 200°C. Data-dependent MS/MS scans were performed in the LTQ for the five most abundance masses with $z > 2$ and intensity higher than 10,000 counts.

Data processing, statistics and ingenuity pathway analysis

LC-MS/MS data files (*.raw) were transferred to Bioworks 3.3.1 data analysis package (Thermo Fisher, San Jose, CA) to identify peptides using the SEQUEST 3.2 search algorithm. The MS/MS spectra were searched against the IPI. HUMAN.v3.83 databases (published time: May 2011) using the following search criteria: maximum allowed missed cleavages for trypsin, 2; fixed modifications of +57.02146 for IAA-modified cysteine residues. Peptide identifications were accepted if they could be established at greater than 95.0% (confidence coefficient) probability as specified by the Peptide Prophet algorithm. The proteins identified with 2 or more peptides were used for the quantitative analysis (PepCount Sequence). The list of differentially expressed proteins was exported to Excel, including protein name and accession number, and then imported to the online software package Ingenuity Pathway Analysis. Core analysis including networks and canonical pathways and biomarker filter analysis were then performed.

Confirmatory experiments by ELISA assay

ELISAs were carried out according to manufacturer's instructions. Human Apolipoprotein A1 ELISA Kit (Uscn Life Science, Inc., Wuhan, China) and Human K-LISA™ mTOR Activity Kit (Calbiochem EMD Chemical, Inc. Merck Biosciences, Germany) were used for in vitro quantitative measurement of APOA1 and mTOR in recollected plasma samples composed of four cervical lesions group (chronic cervicitis, n = 22; HSIL, n = 20; cervical cancer of before stage IIa, n = 20; cervical cancer of after stage IIa, n = 20), respectively. For all ELISA experiments there were three biological replicates. The standard curves were created with APOA1 or mTOR concentration on the *y* axis and average absorbance on the *x* axis. Then the results of concentration were calculated from the standard curves and multiplied by the dilution factor. For comparison of means, ANOVA tests were used as appropriate (SPSS 17.0 software).

Results

Depletion of the highly abundant plasma proteins

The ProteoMiner™ Protein Enrichment Kit Thermo Fisher Scientific, USA is accomplished through the use of a large, highly diverse bead-based library of combinatorial peptide ligands. When complex plasma is applied to the beads, the high-abundance proteins saturate their high-affinity ligands and excess protein is washed away. In contrast, the medium and low abundance proteins are concentrated on their specific affinity ligands. This reduces the dynamic range of protein concentrations while maintaining representatives of all proteins within the original sample. As total mass loads of up to 1.0 mg of protein may be fractionated per analysis, proteins of medium to low abundance will be enriched while more highly abundant proteins that typically mask biomarker discovery are placed into discrete fractions.

2D liquid separation of plasma by chromatofocusing and nonporous, reversed-phase chromatography

The ProteomeLab™ PF 2D system separates peptides and proteins according to their isoelectric points in pH gradient using the chromatofocusing (CF) at 0.3 pH intervals in the first dimension (Figure 1). Each of these pI protein fractions is further separated by hydrophobicity using nonporous silica reverse phase chromatography

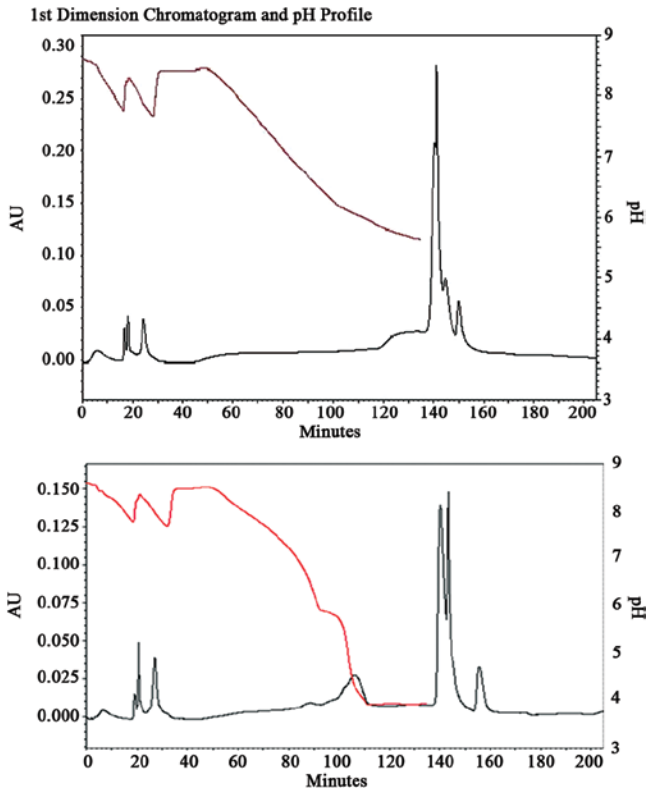

Figure 1. Chromatofocusing HPLC separation. Plasma samples of HSIL (a) and chronic cervicitis (b) were fractionated by isoelectric points in pH gradient 4–8.5 using the chromatofocusing column. Fractions were collected during pH gradient in 0.3 pH intervals or in 1.5 mL volumes when the pH did not change.

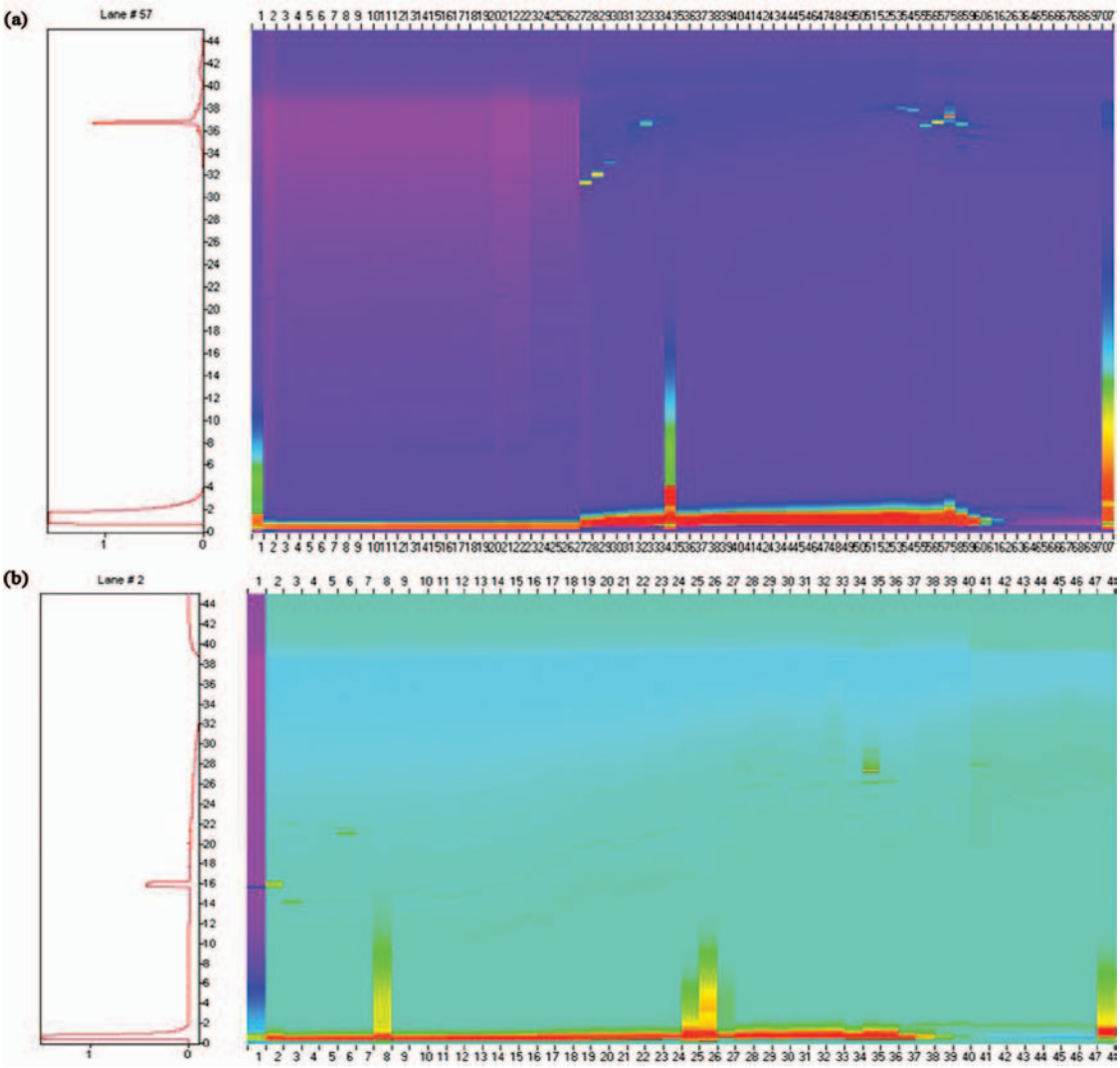

Figure 2. ProteoVue protein expression maps of plasma samples of HSIL (a) and chronic cervicitis (b) Fractions of ~0.3 pH units were collected and are displayed in the *x* axis. Second-dimension elution time is indicated in the *y* axis and corresponds to increasing acetonitrile concentration (0–100% solvent B). Color scale represents the relative intensity of each band based on UV detection at 214 nm.

in the second dimension (Figure 2). A 2D HPLC protein expression map is then generated using ProteoVue software, which conveniently presents the pI of the eluted protein from CF on the *x* axis and the retention time or hydrophobicity from NPS-RP-HPLC of each protein peak on the *y* axis. The ProteoVue software converts the chromatographic UV intensities from the nonporous, reversed-phase chromatography (NPS-RP-HPLC) second-dimension separation of each pI fraction and displays them in a 2D "lane and band" format producing a highly detailed pI versus hydrophobicity protein expression map.

Differential protein expression profiling of plasma samples from HSIL and chronic cervicitis patients by 2D liquid-phase separation

Figure 3 presents 2D HPLC differential display protein expression maps generated using DeltaVue for all three samples. Each plasma sample was separated into 45–50 pI fractions at 0.3 pH intervals from pH 4.0 to 8.5 and then separated by hydrophobicity using NPS-RP-HPLC. The DeltaVue maps compare HPLC chromatograms from different samples with the same pI ranges. This software quantitatively displays one protein map (HSIL) in shades of red and the second map (chronic cervicitis) in shades of green. The difference between the two maps is obtained by point-by-point subtraction or by area difference and displayed as a third middle map. The color (red or green) at a particular location in the difference map indicates which protein is more abundant, and the color brightness indicates the quantitative difference. The program also provides a means to obtain a quantitative number between the expression levels of protein in the two samples. Computer-assisted comparative analysis of the respective peaks' patterns which displayed at least 2-fold change were selected and defined as significant changes. In HSIL versus the chronic cervicitis (Table 2), there were five upregulated peaks (1,2,5,34, and 42) and five downregulated peaks (24,28,29,30, and 33), but in the detection range of pH monitor from pH 8.5 to

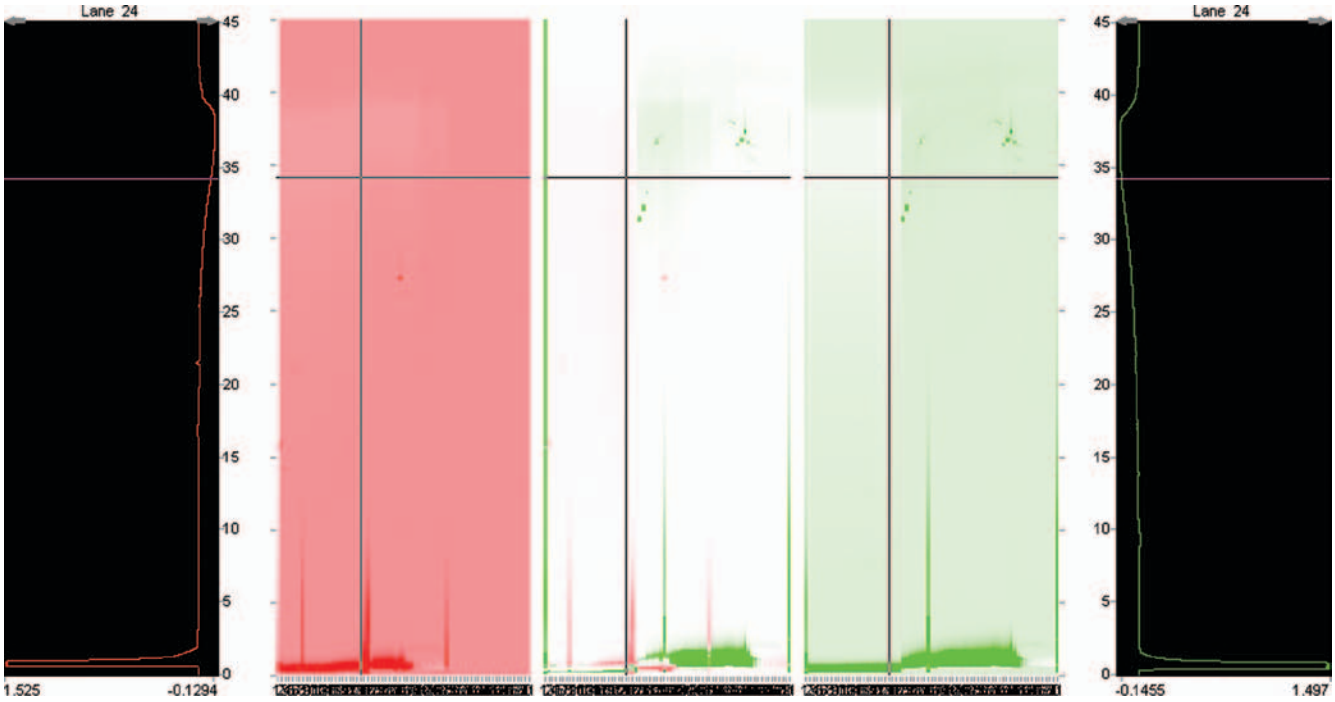

Figure 3. Differential display protein expression maps for two plasma samples. HSIL versus the chronic cervicitis. Differentially expressed proteins are viewed in the center map of each figure panel. The lanes in the center map are obtained by point-to-point subtraction of corresponding data from the maps on either side.

4.0, only three upregulated peaks (1,2, and 5), and one downregulated peak (24) with the red boxes marked.

Identification, biomarker filter and pathway analysis

The four differential expression protein fractions within the detection range (1,2,4, and 24) were subjected to nano-LC–LTQ-MS/MS analysis as described in Materials and Methods section. Identification was based on SwissProt database entries with the SEQUEST search engine 3.2. In HSIL patients and cervicitis control, 31 common differential expression proteins were identified (Supplementary Table S1). The list of result includes the SwissProt accession numbers as well as molecular weight, and pI values, and Unique PepCount and Cover Percent, etc. The accession numbers of the 31 identified differential expression proteins were uploaded into the Ingenuity Pathway Analysis software version 8.7 (http://www.ingenuity.com). We used the tools of the Ingenuity Pathway Core Analyze and biomarker filter to identify biological functions, canonical pathways and candidate biomarker proteins (Supplementary Figures S2–S4). As the result of biomarker analyze, two plasma proteins (APOA1 and mammalian target of rapamycin [MTOR]) as candidate biomarker were screened (Supplementary Figure S5). When classified according to function, inflammatory response, cell to cell signaling and interaction, cellular growth and proliferation were most frequently identified (Supplementary Figure S6). Acute phase response signaling and JAK/Stat signaling and IL-4 signaling, etc. were identified as the canonical pathways that are over-represented in HSIL (Figure 4, Supplementary Figure S3).

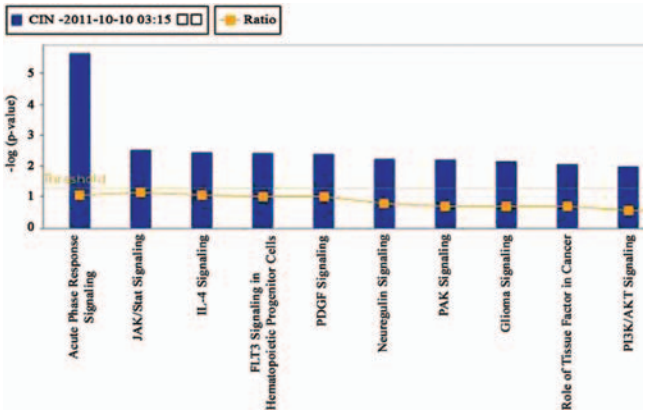

Figure 4. Canonical pathways analysis (top 10).

The validation of ELISA assay

We further tested two proteins APOA1 and mTOR filtered by IPA analysis, for their levels and activity in plasma of four cervical lesions groups in order to validate the changes by ELISA assays. The comparison of ELISA data are shown in Figure 5. APOA1 level was significantly lower in the HISL and cervical cancer of before and after stage IIa groups compared to chronic cervicitis as control ($p = 0.001$). Oppositely, the activity of the mTOR was significantly higher in the HISL and cervical cancer compared to the control ($p = 0.001$).

Discussion

Although cervical cancer is preventable with early detection, it remains the second most common

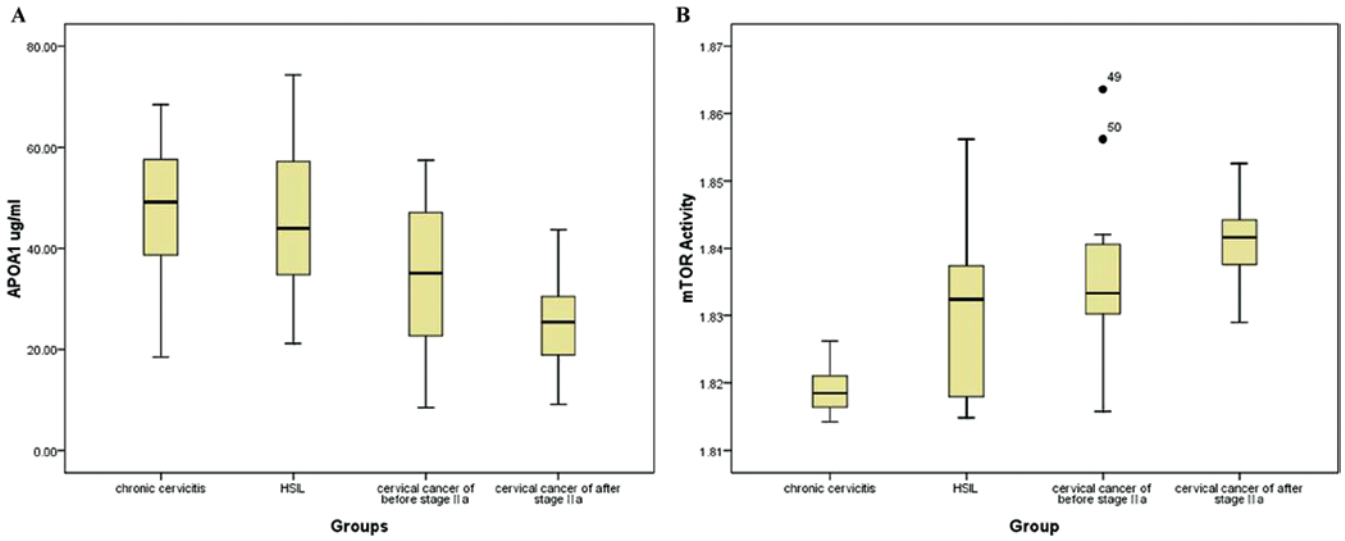

Figure 5. The levels of APOA1 and mTOR are significantly different in HISL and cervical cancer of before and after stage IIa groups compared to chronic cervicitis by ELISA assay. Comparisons of means (ANOVA) were made among four cervical lesions groups. In HISL and cervical cancer, there was a significant decrease of APOA1 and increase of mTOR.

malignancy among women. An understanding of how proteins change in their expression during a particular diseased state such as HSIL will contribute to the ability to predict the occurrence of cervical cancer. Today, the histopathological specificity of cervical cancer biomarkers research has been extensively carrying out for the establishment of early warning system(Xia et al. 2008; Jeong et al. 2008; Looi et al. 2009). With this in mind, we aimed to identify differentially expressed proteins as candidate biomarker in the plasma of HSIL patients using proteomic methods.

In this study, as expected, we have found that compared to the normal control the plasma of patients with chronic cervicitis, the bio-function of differential proteins in HSIL drawn from this comparative analysis of proteomic data was related to acute inflammation response and the multiple cell signal transduction pathways. Dysfunctions of multiple proteins and aberrantly activated signal transduction systems play crucial roles in the development and progression of the disease and vital for the sustenance of cancer. It was demonstrated that in HSIL stage the existence of aberrant signaling systems make for the carcinogenic action, which implies that shutting down the signaling would curb cancerous process. Some of the identified proteins also are important transcription factor, receptor proteins and kinases in blood or secreted and leaked from tumor tissue. As the result, a total of two plasma proteins (APOA1 and mTOR) as candidate biomarker were screened by biomarker filter analysis of the IPA® and verificated by ELISA assay. These two proteins are discussed below.

Apolipoprotein A-I (APOA1) is the major protein component of high-density lipoprotein (HDL) in plasma and an established antiatherogenic factor in lipid metabolism (Wasan et al. 2008; Jayaraman et al. 2011; Flynn et al. 2011). Expression of ApoA1 in arterial endothelium significantly retards atherosclerosis and also isolated as a prostacyclin (PGI2) stabilizing factor, thus may have an anticlotting effect (Yui et al. 1988). But, studies have looked into the association between lipid profiles, using HDL and ApoA1, and cancer risk and suggested APOA1 was downregulation expression in many cancer. For ovarian, breast cancer, prostate cancer and pancreatic cancer, some studies have shown low HDL and ApoA1 levels as well as increased lipid ratios were inversely associated with higher cancer risk and could be one of the biomarkers for early detection (Han et al. 2005; Moore et al. 2006; Chang et al. 2007; Ehmann et al. 2007; Van Hemelrijck et al. 2011). A study by Muntoni et al. compared 519 patients, with any type of solid tumors, and 928 healthy controls and showed that in the cancer group the alterations of lower HDL and ApoA1 and higher triglycerides could be considered as a specific consequence of the presence of a malignant tumor with a diagnostic and prognostic significance using multivariate analysis (Muntoni et al. 2009).

A causal association between downregulation of ApoA1 and risk of HSIL or cervical cancer is thought to be biologically plausible, or the possibility that it was byproducts of the host response to the tumor since ApoA1 and HDL cholesterol have anti-inflammatory properties. Results from the analyses of cholesterol and risk of cancer showed that HDL reduces pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) resulting in reduced tissue damage, reduced infiltration of macrophages and neutrophils, and attenuated tumor formation(Calabresi et al. 2003; Jacobs & Gapstur 2009; Simpson et al. 2010). Thus, HDL and apoA-I reductions may contribute to an inflammatory process that is linked to cervical tumor biology.

The mTOR is serine-threonine kinase that belongs to the phosphatidylinositol 3-kinase (PI3K)-related kinase super-family. In response to extracellular growth factors and nutrients availability, mTOR acts as a key regulator for a number of important cellular functions including cell growth, proliferation, survival, metabolism, and angiogenesis in tumor development and progression (Wullschleger et al. 2006). The perturbations of the oncogenic and tumor suppressors to the signaling network lying upstream of mTOR result in the aberrant activation of this kinase complex and thus the molecular events downstream of mTOR in the majority of human cancers (Azim et al. 2010). Recent studies have found that mTOR activation is sufficient to stimulate an increase in glucose uptake, glycolysis, de novo lipid biosynthesis, promoting protein synthesis and suppressing autophagy, which are considered metabolic hallmarks of cancer, as well as the pentose phosphate pathway (Yecies & Manning 2011). Therefore, our work suggests that the upregulation of mTOR in HSIL patients plasma facilitated neoplastic cells successfully overcome the metabolic demands of an increased rate of growth and proliferation and mTOR could be a potential target for cervical cancer therapeutics. Currently, more than 100 clinical trials target for cancer therapy are ongoing in different phases involving an mTOR inhibitor including breast cancer, nonsmall cell lung cancer, colon cancer and kidney cancer but not cervical cancer (Azim et al. 2010). The upregulation of mTOR in HSIL patients demonstrate that mTOR is an important signaling pathway in the progress of carcinogenesis of cervical lesion and can be effectively targeted and blocked for therapeutic strategies.

Several differences in the plasma proteome between HSIL and inflammatory states were identified; many are unique candidates for active contributors to the generation of HSIL involved in acute phase response signaling and transcriptional signaling pathway, or different adaptor molecules and receptors, crosstalk with other regulatory pathways, and activate or suppress a plethora of kinases that are involved in a multitude of signaling cascades is an important factor in shaping the type, magnitude, and duration of the inflammatory response lead to cytological anomalies or cancer. The pathway and biofunction of the upregulation and downregulation proteins were analyzed using IPA software. The IPA database consists of proprietary ontology representing 300,000 biologic genes, proteins, and molecular and cellular processes. The results of pathways analysis by IPA® revealed that the most statistically significant enriched categories and networks identified were associated with inflammatory response, cell to cell signaling and interaction, cellular growth and proliferation. Canonical pathways involved in acute phase response signaling and JAK/Stat signaling and IL-4 signaling were also enriched.

In classical proteomic research, 2D gel electrophoresis (2DE) is widely used method for separation of complex protein mixtures. However, 2DE has many drawbacks including problematic solubilization and separation of hydrophobic and membrane proteins, limited pI and Mr ranges, narrow dynamic range and poor quantification (Rappsilber & Mann 2002; Patterson 2004). But such in-depth proteomic analyses are formidable challenging due to the complexity and extremely large dynamic range of protein concentrations in plasma. Therefore, it is necessary to reduce sample complexity by either depleting plasma samples of highly abundant proteins with specific antibody-based affinity columns or by multidimensional prefractionation strategies according to protein charge, size or hydrophobicity (Chertov et al. 2004). Recent research demonstrated that 2D HPLC of intact proteins works best for proteins representing extremes in these properties (Sheng et al. 2011). Multi-affinity removal system (MARS) has been a popular method to deplete multiple high-abundance proteins. However, depletion of these abundant proteins can result in concomitant removal of low abundant proteins. In our research, low protein enrichment kit accomplished through the use of a large, highly diverse bead-based library of combinatorial peptide ligands. So, when complex plasma is applied to the beads, the high-abundance proteins saturate their high-affinity ligands and excess protein is washed away. In contrast, the medium and low abundance proteins are concentrated on their specific affinity ligands. As a result of that, high-abundance proteins in our plasma samples had the same concentration, while only low abundance proteins had various contents. It is validated by forward research of many researchers for their effectiveness (Sihlbom et al. 2008; Yadav et al. 2011). Therefore we might conclude it only displayed low abundance proteins could mark disease progress as a biomarker in differential protein expression profiling of plasma samples from cervical squamous cell carcinoma (CSCC) and chronic cervicitis.

We hypothesized that this study could contribute to the discovery of candidate biomarkers for the early detection of HSIL by comparison of protein expressions of plasma specimens from patients with HSIL and with inflammatory benign cervical lesions. And, the further identification of the discovered biomarkers were performed additional confirmation through immunoassay tests. New biomarkers derived from proteomic analysis must be unbiased validated of considerable new recruited clinical samples by prospective collection. Once validated, may provide additional choices in the selection of an optimal panel of boimarkers which would be capable of detecting HSIL in a more general population. Thereby, hastening the identification of novel markers that may contribute to better diagnostic performance is a compulsory task.

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Declaration of interest

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