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RESEARCH

Gelsolin and Ceruloplasmin as Potential Predictive Biomarkers for Cervical Cancer by 2D-DIGE Proteomics Analysis

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Abstract This study aimed to identify candidate proteins which may serve as potential biological markers for cervical cancer using 2D-DIGE. Serum samples of controls, patients with cervical intraepithelial neoplasia grade 3 (CIN 3), squamous cell carcinoma of early (SCC I and II) and late (SCC III and IV) stage were subjected to 2D-DIGE. Differentially expressed spots were identified by tandem mass spectrometry. Validation of candidate proteins in serum and tissue samples were then performed by ELISA and immunohistochemistry (IHC) analysis respectively. A total of 20 differentially expressed proteins were identified. These proteins were found to play key roles in the apoptosis pathway, complement system, various types of transportation such as hormones, fatty acids, lipid, vitamin E and drug transportation, coagulation cascade, regulation of iron and immunologic response. Based on their functional relevancy to the progression of various cancers, 4 proteins namely the complement factor H, CD5-like antigen, gelsolin and ceruloplasmin were chosen for further validation using ELISA. Biological network analysis showed that ceruloplasmin and gelsolin are closely interacted with the oncogene NF-κb. These two proteins were further validated using the IHC. Gelsolin and ceruloplasmin may serve as potential predictive biomarkers for the progression of high grade lesions.

Keywords 2D-DIGE · Cervical neoplasia · Gelsolin · ceruloplasmin · proteomics

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Introduction

Recent advancement in proteomics has provided a powerful tool in the identification of potential biomarker for cancers based on the altered protein expression occurs during carcinogenesis. The current success of the proteomic technology in the development of cancer biomarkers was exemplified by the approval of the ovarian cancer triage test, OVA1, by the USA Food and Drug Administration (FDA) [1]. In the development of the OVA1 test, Fung and colleagues used the SELDI-TOF mass spectrometry technology and identified proteins which provide improvement over CA 125 alone for the detection of early stage of ovarian cancer. Five proteins namely CA125, β2-microglobulin, transferrin, apolipoprotein A1 and transthyretin were combined as a panel and had the ability to discriminate the benign and malignant ovarian tumours. The success of OVA1 diagnostic test clearly demonstrated that the discovery of candidate biomarkers by primary proteomics screening can be translated into clinical application.

Two dimensional fluorescence differential gel electrophoresis (2D-DIGE) technology has been used effectively to detect

more subtle changes in protein expression than conventional 2-DE. It has been used to analyse liver [2] and esophageal cancer tissues [3], colon [4] and lung [5] cancers cell lines, urine of bladder cancer patients [6] and plasma of pancreatic cancer patients [7]. Arnouk et al. [8] employed 2D-DIGE to investigate cervical cancer tissue proteome. They found 8 candidate proteins which could differentiate the normal epithelium from CIN 3 and 4 proteins in differentiating the CIN 3 from cervical cancer [8].

Whilst tissue is the ideal source of cancer biomarkers mining, it has limited utility for screening, early detection and monitoring purposes due to its invasive sampling nature. Hence, non-invasive sampling of serum or plasma holds tremendous potential as a diagnostic source [9]. Each organ and tissue perfused by blood can contribute to the circulating proteins or peptides. Consequently, the serum proteome may reflect the abnormality or pathologic state of organs and tissues.

Compared to other cancers, cervical cancer has an early detection tool since the introduction of the *Pap* smear in 1941. In developed countries, the *Pap* smear screening program has reduced cervical cancer deaths by 70 % [10]. The *Pap* smear remains an effective and widely accepted screening program for early detection of cervical cancer. However *Pap* smear alone is insufficient for monitoring disease, treatment and the prognosis of cervical cancer. The premalignant lesions of cervical cancer show a greater individual variability. Most cases of CIN 1 and a large proportion cases of CIN 2 and CIN 3 have the potential to spontaneously regress [11]. There are only a few percent of CIN 1 and CIN 2 lesions and only a slightly larger minority (12 to 32 %) of cases with CIN 3 which will progress to invasive cancer if left untreated [11]. Thus, it is important to treat the CIN appropriately since there is risk of developing cancer. The decision to surgically remove the CIN lesion is difficult since only a small percentage of CIN cases will develop into invasive cancer [12]. This emphasizes the need for predictive biomarkers which could differentiate the individual pattern of CIN with high risk of progression into invasive cancer that would be valuable in deciding the treatment option. Besides, the biomarker can also be used to monitor the progression of disease and the effectiveness of the treatment. Such biomarker(s) can be combined with the existing screening method and applied as an ancillary test rather than replacing the current screening program.

In the present study, we employed the 2D-DIGE system to examine the differential protein expression in the serum of patients with CIN 3 and SCC of cervix. Compared to the conventional 2DE, 2D-DIGE based proteomics with fluorescence labelling has higher sensitivity, reproducibility and allow for more accurate quantitative and qualitative analysis with less technical variation with the internal standard. In addition, 2D-DIGE has the potential to detect and quantify patterns of protein expression profiles as with SELDI-TOF technology [13].

Besides identifying the differentially expressed proteins, this study also focused on the analysis of biological pathway related with the identified proteins by using Metacore™ software. The major proteins found to be related in relevant carcinogenesis pathways were validated by ELISA and IHC. Identification of differentially expressed proteins and further biology network analysis may lead to greater understanding of the role of the proteins in different biological pathways involved in the carcinogenesis of cervical cancer and may contribute to the development of novel biomarker(s) for cervical cancer.

Materials and Methods

Sample Collection and Purification of Serum

The study was approved by the Institutional Ethics Committee of the university. Patients were recruited from Obstetrics and Gynaecology clinic with signed informed consent. A total of 152 serum samples were collected from newly diagnosed patients with CIN (n=57), SCC (n=46) and adenocarcinoma (n=11). The patients had not received any prior treatment and their cervical lesions were confirmed by *Pap* smear, colposcopy observation and punch biopsy. The normal controls (n=38) were those who came for routine screening and had normal *Pap* smears. Of 152 samples, 28 samples were randomly selected for 2D-DIGE analysis with each of the 4 study groups (normal control, CIN 3, early stage and late stage of SCC) comprising of 7 samples. Validation was performed on all 152 serum samples by ELISA.

A total of 126 formalin fixed paraffin embedded cervical tissue blocks were retrieved from the archive of the Histopathology Unit for the IHC analysis. Data on the grade of differentiation and lymph node involvement status of SCC were obtained from the Medical Record Unit. There were 30 normal cervical tissues, 49 cases of CIN, 41 cases of SCC and 6 cases of adenocarcinoma.

Albumin and immunoglobulin G (IgG) from the serum samples were depleted using an albumin and IgG depletion spin trap (GE Healthcare, UK) to improve the detection of low abundance proteins. The depleted serum samples were then mixed with 4 volumes of pre-cooled acetone and incubated for 2 h at −20 °C. The protein pellets were obtained after centrifugation at 13 000 g for 10 min at 4 °C. The pellets were then dried at room temperature for 5–10 min and reconstituted with a sample preparation buffer (8 M urea, 4 % CHAPS).

Labelling of Cy dye and 2-dimensional Separation

The pH of the serum protein was adjusted to 8.5 by 50 mM sodium hydroxide. The concentration of the protein was further

adjusted to 10 μg/μl with DIGE lysis buffer (30 mM Tris, 7 M urea, 2 M Thiourea, 4 % CHAPS). Then, 30 μg of protein from each of the 28 samples were pooled together as the internal standard. A total of 60 μg of protein of each samples were randomly labelled with 1.2 μL of 400pmol Cy3 or Cy5 and the internal standard was labelled with Cy2. The labelling reaction was performed for 30 min on ice in the dark. The reactions were then quenched by adding 10 mM lysine for 10 min on ice in the dark. Then, 50 μg each from Cy3 and Cy5 labelled samples were combined with 50 μg of Cy2 labelled internal standard. An equal volume of DIGE 2X sample buffer (8 M Urea, 4 % CHAPS, 1.5 % IPG buffer,130 mM DTT) was added to each mixed sample and the total volume was made to 340 μl with rehydration buffer (8 M urea, 2 % CHAPS, 2 % IPG buffer pH 3–10, DTT 2.8 mg/ml).

Samples were then rehydrated into 18 cm IPG strips pH 4–7 (GE Healthcare, UK) for 20 h at room temperature in the rehydration plate (GE Healthcare, UK). The isoelectric focusing were carried out for total of 100 kVhrs at 20 °C and 50 μA per IPG strip in dark. After the isoelectric focusing, the gel strips were equilibrated in equilibration buffer (6 M urea, 50 mM Tris –HCl pH 8.8, 30 % glycerol, 2 % SDS) supplemented with 65 mM dithiothreitol (DTT) for 15 min with gentle shaking followed by 100 mM iodoacetamide (IAA) in equilibration buffer for another 15 min again with gentle shaking. The equilibrated strips were then loaded on top of 10 % SDS-polyacrylamide gels that had been pre-casted in low fluorescence glass plates. The second dimension separation was then carried out at 20 W/gel using Ettan Dalt six electrophoresis system (GE Healthcare,UK). After the 2DE separation, the gels were scanned using the EDI scanner (GE Healthcare, UK). The images were then analysed using the DeCyder™ 2-D Differential Analysis Software (DeCyder 2D) V8.0. Protein spots that were differentially expressed in different study groups with p values ≤ 0.05 were marked and selected for protein identification.

In Gel Digestion

The gels were subjected to silver staining for spot visualization and picking. Spots of interest were excised and destained with 15 mM potassium ferricyanide and 50 mM sodium thiosulphate. The spots were then reduced in 10 mM DTT followed by alkylation in 55 mM IAA. After being washed with 50 % acetonitrile (ACN) and dehydrated in 100 % ACN, the gel fragment was dried in a speed vacuum. The dried gel fragment was then digested with 7 ng/μl trypsin (Promega, CA, USA) overnight at 25 °C. The digested proteins were extracted twice with 50 % ACN and followed by 100 % ACN and kept at −80 °C until further analysis by LC-MS/MS.

LC MS/MS Analysis

The chromatography separation was performed using Nano-Ultra Performance Liquid Chromatography (Nano-UPLC) (Waters, USA). The protein extract in ACN was dried in speed vacuum before being reconstituted with 0.1 % formic acid in water and inserted into the autosampler *Nano Acquity UPLC™*. Peptides were separated on a reverse phase column using a binary solvent system made up of solvent A (0.1 % formic acid in water) and solvent B (acetonitrile in formic acid) with a total runs time of 45 min.

Q-Tof Premier™ Mass spectrometry was programmed to step between normal (5ev) and elevated (25 to 40ev) collision energy on the gas cell using a scan time of 1.05 per function over 50 to 1990 m/z. Combined peptide mass spectra and MS/MS data were then collected and submitted for database searching in *Uniprot* databases.

The identified proteins were subjected to network analysis using the network building tool MetaCore™ (GeneGo, Thomson Reuters, USA). The differentially expressed proteins in this experiment and the proteins from MetaCore database were used to generate networks which showed the possible interaction between these proteins in different biological pathways.

ELISA

ELISA were conducted to confirm the differential expression of gelsolin, ceruloplasmin, complement factor H (CFH) and CD5-like antigen (CD5L). The ELISA analysis was performed according to manufacturer's (Cusabio Biotech, China) protocol.

Immunohistochemical Staining

The sequential tissue sections (3 μm) were mounted on poly-L-lysine-coated slides. The slides were dewaxed by gradual washings in xylene and then dehydrated in various concentrations of alcohols (100 %, 90 %, 70 % and 50 %). Slides were then incubated in 3 % hydrogen peroxide in distilled water to quench endogenous peroxide activity, after which the slides were washed in running water. Antigen retrieval was performed by incubating slides in Target Retrieval Solution (TRS) pH6 for 1 min. The sections were the washed with Tris-buffered saline (TBS) pH7.6. Detection of gelsolin and ceruloplasmin was performed using monoclonal antibody anti-gelsolin (rabbit monoclonal antibody ab75832: Abcam, Cambridge, UK) and polyclonal antibody anti-ceruloplasmin (rabbit polyclonal antibody ab48614: Abcam, Cambridge, UK) respectively. Slides were incubated with anti-gelsolin antibody (at dilution of 1:250) and anti-ceruloplasmin (at dilution of 1:30) for 30 min at room temperature.

After washing in TBS, the sections were incubated with the Dako Real Envision detection system (Dako, Glostrup, Denmark) for 30 min. Staining was completed after 5 min of incubation with a freshly prepared 3, 3′-diaminobenzidine (DAB). Sections were then counterstained with hematoxylin, followed by rehydration and mounted with DPX for microscopic viewing. Colon and kidney tissues were used as positive controls for anti-gelsolin and anti-ceruloplasmin respectively. The negative control was obtained by substituting the primary antibody with TBS.

The immunoreactivities of gelsolin and ceruloplasmin were observed in the cells with distinct brown staining confined to the cytoplasm for both antibodies. The percentage of positive tumour cells was graded according to Watari et al. [14] with some modification. The staining extent was scored 0 (negative) when none or <10 % of tumour cells were stained; as +1, when 10–25 % cells were stained; as +2, when 26–50 % tumour cells were stained and as +3 when >50 % cells were stained.

Statistical Analysis

The serum level of protein expression between different groups of study in ELISA experiment were compared using one-way ANOVA test. Association between immunohistochemical scores and clinicopathological variables (stage of cancer, grade of differentiation and lymph node involvement) of tissue specimens were evaluated by the Chi square (χ^2) test. All statistical analysis was performed with SPSS 16.0 (SPSS Inc, Chicago, Illinois, USA) and p values ≤0.05 were considered statistically significant.

Results

A total of 1974 differentially expressed protein spots were identified and 45 of them were found to be significantly ($p \leq 0.05$) altered in their abundance between groups. A total of 20 proteins were successfully identified from the 45 spots by LC-MS/MS. The identified proteins were grouped based on their function (Table 1). They were found to be involved in the apoptosis pathway, transportation of hormones, fatty acids, lipid, Vitamin E and others, coagulation cascade, complement system, transportation and regulation of iron and immune response. Two uncharacterized proteins and 3 proteins with unknown function were also identified.

Previous reviews of these proteins had revealed that the complement factor H (CFH), CD5-like antigen (CD5L), gelsolin and ceruloplasmin were involved in the carcinogenesis of various cancers but the expression of these proteins have not been well studied in cervical cancer. Hence, these 4 proteins were chosen for further validation using the ELISA test.

ELISA analysis revealed that the serum levels of CFH (Fig. 1a) and ceruloplasmin (Fig. 1b) were significantly increased and the level of gelsolin (Fig. 1c) was decreased in the SCC cases compared to normal controls in concordance to the 2D-DIGE results. The serum level of CD5L (Fig. 1d) was also elevated in CIN and SCC cases compared to normal controls but did not correlate with 2D-DIGE analysis.

Protein network analysis found gelsolin and ceruloplasmin interacted with NF-κB (Fig. 2). The differential expressions of these two proteins in cervical neoplasia were further validated using IHC analysis.

Gelsolin showed negative immunoreactivity in the normal cervical squamous epithelium (Fig. 3a). All CIN 1 and CIN 2 cases showed negative staining of gelsolin except for one CIN 2 case which exhibited positive staining. All CIN 3 cases with invasion into stroma or glands were found positively stained with gelsolin in the cytoplasm of dysplastic cells (Fig. 3b and c). The CIN cases exhibited staining scores of +1 or +2. Seventy percent (29/40) of SCC cases stained gelsolin positively in the cytoplasm of tumour cells (Fig. 3d). The normal endocervical glands and adenocarcinoma cells also exhibited positive immunostaining for gelsolin (Fig. 3e and f). However, the adenocarcinoma cells exhibited less intense immunostaining of gelsolin compared to normal endocervical glands. The immunostaining of gelsolin showed discrepancy with the 2D-DIGE and ELISA results. No significant correlation was found between the gelsolin immunoreactivity with lymph node involvement (p=0.540) and cancer differentiation grades (p=0.278).

Ceruloplasmin showed negative immunoreactivity in normal squamous epithelium (Fig. 4a), endocervical glands of cervix (Fig. 4b) and CIN 1 cases. The immunoreactivity of ceruloplasmin was distributed in the all layers of CIN 3 (with and without invasion) with staining scores of +1 and +2. About 80 % of the late stage SCC cases showed stronger (score 3+) immunostaining of ceruloplasmin than in the early stage of SCC and adenocarcinoma (majority scored 1+ and 2+). The immunostaining results of ceruloplasmin showed were consistent with the 2D-DIGE results. SCC immunoscoring was not correlated with lymph node involvement (p=0.309) and tumour differentiation grade (p=0.451).

Discussion

Previous studies on cervical cancer proteome were aimed at investigating the general proteomics profile of the serum or plasma from cervical cancer patients [15–17] and the anticancer effects of drugs on protein expression changes [18, 19]. Studies that compare the cervical cancer proteome with cDNA microarray data are still lacking. Clearly, proteomics-based cervical cancer biomarker discovery is still at the early stage. Here, we investigate the serum proteomic profiles of pre-neoplasia

Table 1 Differentially expressed proteins in serum of cervical neoplasia patients identified by LC-MS/MS

Protein name and known function	*Uniprot* ID	Covarage (%)	Observed value		Theoritical value		Avarage ratio[a]					
			MW (KDa)	p*I*	MW (KDa)	p*I*	CTR VS CIN 3	CTR VS Early	CTR VS Late	CIN3 VS Early	CIN3 VS late	Early VS Late
Apoptosis												
1 CD5 like-Ag	O43866	68.58	45.97	5.99	38.06	5.13	1.48	1.19	−1.2	−1.24	−1.78	−1.44
2 Gelsolin	P06396	44.68	79.59	7.06	20.77	4.48	1.13	−1.19	−1.47	−1.35	−1.67	−1.24
Complement system												
3 Complement factor H	P08603	58.57	91.63	6.28	51	6.71	1.42	1.13	1.03	1.25	1.46	1.17
4 Complement factor B	P00751	31.28	91.63	7.42	85.47	6.65	−1.21	−1.51	−2.23	−1.25	2.69	3.37
5 protein C1S	P09871	28.23	81.17	4.98	75.85	4.9	−1.24	−1.47	−1.29	−1.18	−1.04	1.14
Transportation												
6 Isoform 2 of Serum albumin	P02768 2	32.58	46.12	6.27	47.32	5.92	1.16	1.15	−1.43	−1.01	−1.65	−1.64
7 Afamin	P43652	29.38	78.13	5.53	69.02	5.53	−1.14	−1.25	−1.27	−1.09	−1.11	−1.02
8 Apoplipoprotein A1		79.02	23.67	5.77	30.75	5.43	−1.2	−1.08	−1.59	−1.07	−1.58	1.48
Coagulation cascade												
9 Beta 2 glycoprotein	P02749	36.81	59.34	7.42	38.27	7.86	−7.37	−1.44	−5.24	5.12	1.41	−3.64
10 Alpha 1 antitrypsin	P01009	55.55	58.76	4.00	34.73	4.86	1.06	−1.21	1.08	−1.15	1.14	1.31
Transportation & regulation of iron												
11 Serotransferrin	P02787	51.71	72.86	7.72	76.99	6.75	1.15	1.05	−1.46	−1.1	−1.27	−1.4
12 Hemopexin	P02790	43.07	70.36	5.97	51.64	6.57	−1.1	1.01	1.31	1.1	1.44	1.3
13 Haptoglobin	P00738	39.65	45.86	4.99	45.17	6.12	1.07	1.46	1.31	1.37	1.23	1.11
14 Ceruloplasmin	P00450	23.38	85.73	5.72	122.12	5.33	−1.21	−1.02	1.25	1.18	1.51	1.27
Immunologic reaction												
15 Ig mu heavy chain disease protein	P04220	26.34	46.98	7.44	43.03	4.95	1.37	1.12	−1.42	−1.22	−1.95	−1.6
Unknown function												
16 cDNA FLJ42001 fis clone	Q6ZVW3	25.86	46.06	6.16	15.63	10.7	1.67	1.61	1.08	−1.04	−1.49	−1.55
17 PRO2275	Q9P173	35.83	81.17	4.98	13.08	9.58	1.3	1.49	1.64	1.14	1.26	1.1
18 Transferrin variant Fragment	Q53H26	24.92	43.62	7.59	77.03	6.63	−1.03	−1.06	−1.47	−1.03	−1.42	−1.39
19 uncharacterized protein	-	31.82	51.72	6.22	45.06	5.72	−1.64	−2.17	−1.74	−1.32	−1.06	1.24
20 uncharacterized protein	-	44.68	14.4	6.68	5470	9.42	−3.21	1.12	2.45	3.6	2.19	7.86

[a] Average ratio is ratio of the different protein abundance between two groups. The (+)indicating increasing in protein expression and the (−) sign indicating decreasing in the protein expression

(CIN) and of cervical cancer by 2D-DIGE. Comprehensive validation analysis was performed using a large cohort of samples by ELISA and IHC.

We identified 20 differentially expressed proteins in the serum of CIN3 and SCC cases. The biological pathway analysis of these proteins classified them into different functional groups, i.e. apoptosis, complement system, regulation and transportation of iron, coagulation cascade, various types of transportation such as the lipid, fatty acid, hormones and vitamin E transportation and immunologic response.

Apoptosis is a fundamental process in the normal development and tissue homeostasis. Failure in apoptosis can lead to diseases including cancers. Our results showed differential abundance level of gelsolin and CD5L proteins, both of which are involved in the apoptosis pathway [20, 21]. Gelsolin is an actin-binding protein that is a key regulator of actin filament assembly and disassembly [22]. In this current study, we observed the down-regulation of full length (~80Kda) gelsolin in CIN 3 and SCC of cervix by 2D-DIGE. Previous studies had revealed the dual roles of gelsolin as a tumour enhancer [20] or a tumour suppressor [23] depending on the type of cancer. Koya et al. (2000) reported the up-regulation of full length gelsolin playing a role as a tumour enhancer in acute T-cell leukaemia. It inhibits the apoptotic cell death via the inhibition of the cytochrome C release from mitochondria resulting in the lack of activation of caspases [20]. In contrast, Fujita et al. (2001) found that the full-length gelsolin may act as a tumour suppressor by reducing the

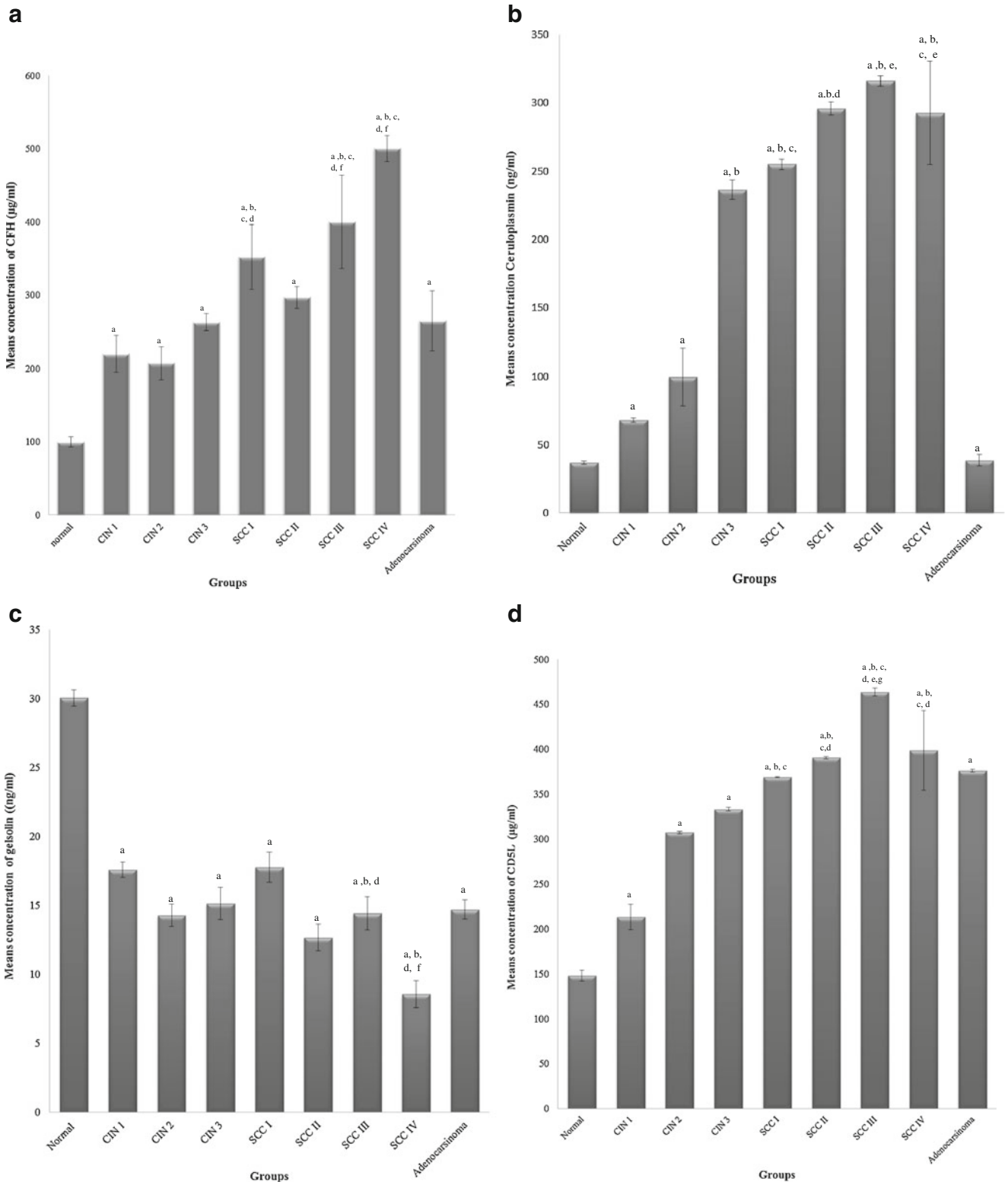

Fig. 1 Quantitative comparison of **a** CFH, **b** ceruloplasmin **c** gelsolin and **d** CD5L, and in serum of normal control, CIN, SCC and adenocarcinoma of cervix. The significant difference between groups denoted by **a** when compared to normal control, **b** when compared to CIN, **c** when compared to CIN 2, **d** when compared to CIN 3, **e** when compared to SCC I, **f** when compared to SCC II and **g** when compared to SCC IV, found by one-way ANOVA test. Data presented in mean±SEM

chemotactic cell migration to fibronectin in B16-BL6 melanoma cells. They also found the retardation of tumour growth and suppression of lung metastasis in vivo with clones expressing full-length gelsolin [23]. They suggested that the

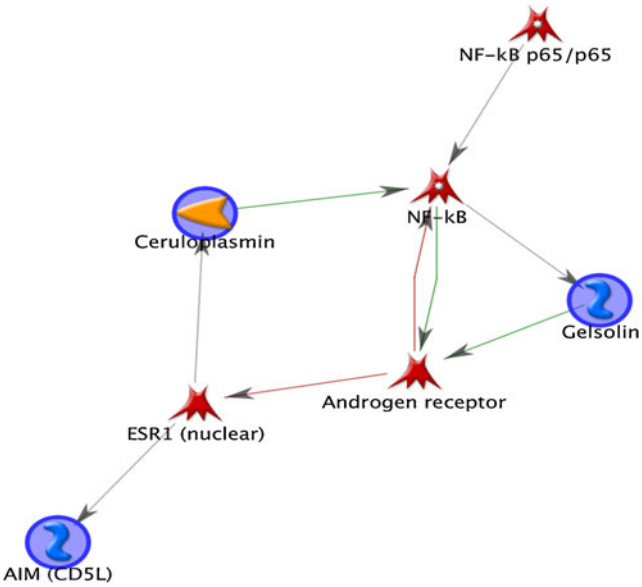

Fig. 2 Protein interaction network of gelsolin and ceruloplasmin with NF-κB. The green error indicated the activation process, black error indicated transcription regulation and the red error indicated binding of the molecules

carboxyl-terminus of gelsolin could have a critical role in the suppression of chemotactic cell migration and metastasis although the details of the molecular mechanism(s) for tumour suppression by the full-length gelsolin remain to be elucidated [23].

We found increased expression of gelsolin in the tissue of SCC of the cervix which contradicted with the serum proteomics results. The discrepancy in the results may be due to the presents of different isoforms of gelsolin. Gelsolin has been shown to exist in two isoforms, 80 kDa cytoplasmic protein and 85 kDa plasma gelsolin [24]. Plasma gelsolin is the only isoform that is secreted into extracellular space and it differs from cytoplasmic gelsolin by addition of 25 amino acids peptide at its NH_2 terminus and is more positively charged [25]. In our study, we have identified 79.59 kDa (p*I* 7.06) gelsolin in the serum by 2D-DIGE. However we were not able to characterize whether the identified protein is cytoplasmic or plasma gelsolin. On the other hand, we proposed that the gelsolin identified in the tissue of cervix by IHC is the cytoplasmic isoform.

In contrast to SCC tissues, the adenocarcinoma tissues exhibited lower expression of gelsolin than normal endocervical glands and SCC. This further suggests the possibility of dual roles of gelsolin depends on histologic type of cervical cancer. In addition, we found gelsolin highly expressed in the high grade intraepithelial lesions (CIN3) with invasion into either stroma or endocervical glands. The positive association between gelsolin expression and invasiveness of malignant cells has been detected in several cancers [26, 27]. Previous studies suggested that gelsolin may be involved in the modulation of several signaling pathways such as the Ras

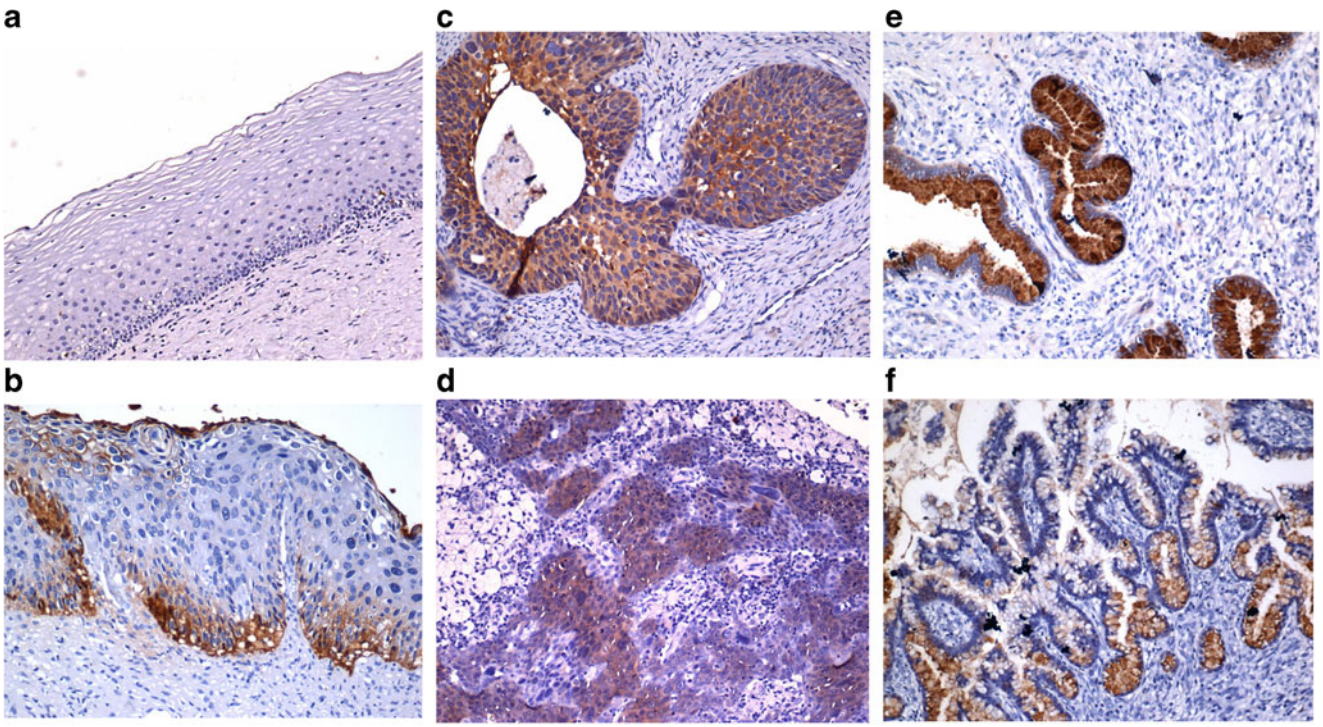

Fig. 3 Immunoexpression of gelsolin in cervical tissues at X 200 magnification. **a** Negative staining of gelsolin in normal squamous epithelium. **b** Positive staining of gelsolin in CIN 3 with stroma invasion **c** Positive staining of gelsolin in CIN 3 with endocervical gland invasion **d** Positive staining of gelsolin in SCC tissue **e** Normal epithelial of the cervical glands showed cytoplasmic staining of gelsolin **f** Adenocarcinoma showed positive expression of gelsolin

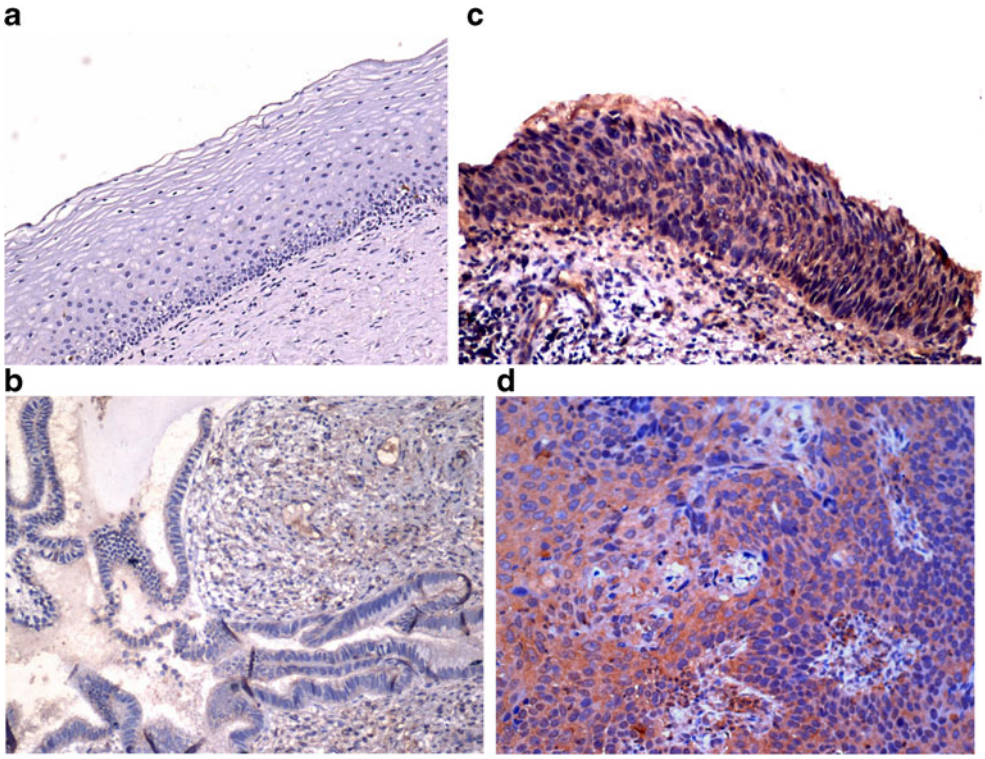

Fig. 4 Immunoexpression of ceruloplasmin in cervical tissues (X 200 magnification). Negative staining of ceruloplasmin in normal squamous epithelium (**a**) and normal cervical glands (**b**). Positive staining of ceruloplasmin in CIN 3 (**c**), SCC tissue (**d**) and adenocarcinoma (**e**)

oncogenic signalling pathway (Ras-P13K-Rac) [28], phospholipase C [29] and phosphatidylinositol 3-kinase [30] pathways results in altered actin cytoskeletal architecture and increased cell motility which could facilitate the invasion of tumour cells. It is postulated that gelsolin may promote the invasion of tumour cells and may serve as a valuable tissue marker in distinguishing the patients with high risk of progression of premalignant cells into invasive cancer.

The other apoptosis related protein, CD5L also known as Sp alpha, is a soluble protein belonging to group B of the scavenger receptor cysteine rich (SRCR) [31]. It was found to play an important role in the regulation of innate and adaptive immune systems [32]. Increased levels of serum CD5L have been found in the liver fibrosis [31], liver cirrhosis, hepatocellular carcinoma [33] and lung cancer [21]. The exact mechanism of CD5L in tumorigenesis is still unclear. However, a previous study suggested that CD5L may promote the malignant transformation by blocking a mechanism of epithelial apoptosis that would normally support immunosurveillance [21]. We found up-regulation of CD5L in CIN 3 and early stage of SCC and the expression was further confirmed by the ELISA but it was found to be down-regulated in the late stage of SCC although ELISA failed to confirm this.

In this study, we observed discrepant protein expression results between proteomics analysis and ELISA or IHC. This may be due to the loss of proteins that may have occured during the sample preparation before 2D-DIGE analysis. The probability of removing some low abundance proteins along with the high abundant proteins is a drawback of the protein depletion method. The albumin is the most abundance protein in the human serum. It binds and transports various compounds including hormones, lipid and amino acids. Thus, the removal of albumin from serum may also lead to the specific loss of some low abundance proteins which may confound subsequent proteomic analysis.

Complement cascade has been related to cellular killing and inflammation process [34]. However, recent findings suggested that the complement system may play a role in tumorigenesis of various cancers by facilitating the cellular proliferation [34, 35]. The complement system consists of a cascade of proteins that are responsible in cell lysis process [36]. The complement activation occurs on the surface of target cells and is initiated by the binding of one or more molecules of the classical, alternative or mannose-binding lectin pathways [34]. Our proteomic data found differential expression of two proteins; complement factor H (CFH) and complement factor B (CFB), which are involved in the alternative complement pathway of the complement cascade. Our data demonstrated up-regulation of CFH in the serum of SCC patients and this observation was further confirmed by ELISA analysis. CFH is a 150 kDa glycosylated plasma protein which is the main inhibitor of the alternative pathway of complement cascade [37]. The overexpression of CFH has been demonstrated in several malignancies such as ovarian [38], bladder [39] and lung cancers [36]. In contrast to CFH, we found down-regulation of CFB in the serum of SCC patients in 2D-DIGE. CFB is component of the alternative

pathway of complement activation. Upon activation of the alternative pathway, CFB will be cleaved by complement factor D yielding non-catalytic chain Ba and catalytic chain Bb [40]. Bb is involved in the proliferation of pre-activated B lymphocytes, while Ba inhibits their proliferation [41]. The over-expression CFB has been shown in breast [42] and pancreatic [43] cancers. However, we found down-regulation of CFB in SCC, which is similar to that reported by Seriramalu et al. [44].

The altered iron intracellular homeostasis and perturbations in the functioning of proteins involved in the iron regulatory pathways have been found to be associated with cancer progression [45, 46]. Although we have identified several proteins related to iron regulation, our focus was on the ceruloplasmin as serotransferrin, hemopexin and haptoglobin are abundant proteins in the serum which are not a suitable candidate biomarkers for cancers. Ceruloplasmin is a copper-binding protein that oxidizes iron (Fe^{2+}) into a transportable and usable state (Fe^{3+}) allowing cells to take up the iron for cellular proliferation [47]. It is also found to play a role in the angiogenesis process via stimulation of endothelial cells [48] when copper is bound to the peptides [47].

Elevated levels of ceruloplasmin were identified in the serum [49, 50] and tissue [50] of several malignancies and found associated with poor prognosis of cancers [50, 51]. In accordance to these findings, we found ceruloplasmin expression was upregulated in the serum and tissues of cervical SCC and adenocarcinoma of the cervix. Furthermore, our immunohistochemical analysis of ceruloplasmin expression in normal cells and neoplasia lesions showed that it was not expressed in normal squamous epithelium and endocervical glands; suggesting the probability that this protein is synthesized by neoplastic cells to provide sufficient iron for the actively proliferating tumour cells.

In this study, we also found deregulation of coagulation related proteins such as β-2 glycoprotein and α-1 antitrypsin in the serum of cervical SCC patients. This might be related to some degree of coagulation that often occurs in cancer patients [52]. Our proteomics data demonstrated down-regulation of β-2 glycoprotein and up-regulation of α-1 antitrypsin in CIN 3 and SCC. However the role of these proteins in the oncogenesis remains unclear. The coagulation factors could have direct tumour stimulating effects and their levels may not only reflect coagulation but also the biology of the underlying cancer [52] .

The molecular pathway analysis of identified proteins showed a close interaction between gelsolin and ceruloplasmin with NF-κB. NF-κB is involved in the signalling pathways that regulating immune response and pro-inflammatory reactions [53]. It is also found to play a role in regulating the expression of genes which are involved in the development and progression of cancer such as proliferation, migration and apoptosis [53]. The activation of NF-κB by various stimuli, including tumour necrosis factor (TNF), stimulates the production of gene products that keep the cells proliferating and protect the cells from apoptosis [54]. There are limited studies investigating the relationship between ceruloplasmin and gelsolin with NF-κB. Persichini et al. [55] found a number of putative binding sites for NF-κB transcription factor on the ceruloplasmin promoter and reported that the expression of ceruloplasmin to be regulated by IL-1β after activation of NF-κB in angiogenesis. In addition, nucleic acid sequence analysis of the gelsolin promoter also identified putative transcription binding site for NF-κB [56]. However the exact mechanism behind the correlation between these proteins is still unknown. It is clear from our study that there is a need for further functional studies elucidates the involvement of ceruloplasmin and gelsolin in the activation of NF-κB in the progression of cervical cancer which may lead to a better understanding of the molecular mechanisms involved in the progression of cervical cancer.

In this study, we also attempted to correlate the candidate proteins expression with clinicopathology parameters such as lymph node involvement and tumour differentiation grade. However, both gelsolin and ceruloplasmin expressions failed to show any significant correlation with lymph node involvement and tumour differentiation grade in the cervical cancer cases studied. A previous study also showed a negative correlation between the gelsolin expression and lymph node involvement in cervical cancer [57]. However, the gelsolin expression showed a positive correlation with lymph node involvement in pancreatic cancer and was reported to be facilitating the metastasis of the cancer [58]. The expression of ceruloplasmin has also been reported to correlate with lymph node involvement and facilitate in the metastasis of gastric cancer [59].

Conclusion

Our study demonstrated that gelsolin and ceruloplasmin may not be indicators of tumour metastasis and candidate prognostic biomarkers for cervical cancer. However, we suggest that gelsolin and ceruloplasmin may be used as predictive biomarkers for the progression of high grade intraepithelial lesions to cervical cancer due to their distinctive expressions in microinvasive and cancerous lesions of the cervix.

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