Analysis of protein profiles in human epithelial ovarian cancer tissues by proteomic technology

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Summary

Background: Screening in ovarian cancer is progressively finding out candidate genes and proteins which may work as screening biomarkers and play a role in tumor progression. We examined the protein expression patterns of ovarian cancer tissues using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of fight mass spectrometry (MALDI-TOF MS). *Methods:* Tissues from 36 ovarian cancers and 20 normal ovaries were examined by 2-DE. The images of silver stained gels were analyzed by ImageMaster 2D Elite. The peptide mixtures, after in-gel digestion, were determined by MALDI-TOF MS for fingerprinting. The de-isotope tryptic peptide profiles were matched by using the Mascot search engine based on the entire NCBI and Swiss-Prot protein databases. Western/dot blots were then applied to verify the findings. *Results:* In ovarian cancer, 12 proteins that showed differential expressions were identified unequivocally. Among these proteins, five proteins (galectin-1, cathepsin B, ubiquitin carboxy-terminal hydrolase L1, HLA class II antigen DRB1-11 and heat shock protein 27) were up-regulated and seven proteins (cellular retinol-binding protein, transthyretin, SH3 binding glutamic-rich-like protein, tubulin-specific chaperone A, DJ-1, gamma-actin and tropomyosin 4) were down-regulated. *Conclusion:* The present study is the first to report the up-regulation of ubiquitin carboxy-terminal hydrolase L1 and the down-regulation of SH3 binding glutamic-rich-like protein, tubulin-specific chaperone A, and tropomyosin 4 in human ovarian cancer tissues. Further cloning and functional analysis of these salient proteins will provide more information on their pathophysiologic roles in ovarian cancer.

Key words: Ovarian cancer; Proteomics; Two-dimensional gel electrophoresis; Matrix-assisted laser desorption/ionization-time of fight mass spectrometry.

Introduction

Ovarian cancer is the most lethal gynecological malignancy with a 5-year survival rate of about 30% [1]. The high death rate is due to late stage of presentation and lack of reliable biomarkers for detection of early-stage cases. CA-125, the currently best characterized serum marker for epithelial ovarian cancer, lacks the sensitivity for detecting early-stage disease, as only 50% of early-stage cases have elevated serum levels [2, 3].

Based on advances in automation and bioinformatics, a new discipline of biology, proteomics, has emerged. Proteomics refers to the study of all the protein forms expressed within an organism as a function of time, age, state, external factors, etc. [4, 5]. From the biomedical standpoint, the field of proteomics has a greater potential because the identified proteins are the biological end-products [6]. Recently, proteomic approaches to identify tumor markers of ovarian cancer are undergoing. Alaiya and coworkers reported the protein expression patterns in primary carcinoma of the ovary [7]. Another published study reported the proteins that were differentially expressed between benign, malignant, and normal ovaries [8].

Despite enormous efforts, relevant markers useful for screening have not been established in ovarian cancer. In this study, by using two-dimensional gel electrophoresis (2-DE) and matrix-assisted laser desorption/ionization-time of fight mass spectrometry (MALDI-TOF MS), we examined the protein expression profile difference in human ovarian cancer tissues and normal ovaries.

Materials and Methods

Collection of tissue samples

Tissue specimens were obtained during the surgery from the National Taiwan University Hospital (NTUH) according to the standard procedures. Ovarian tissues from cases of uterine myoma were used as normal controls. The Ethics committee of the NTUH approved the study protocol and all subjects gave informed consent before participation in the study. Each case of ovarian cancer was also evaluated by H&E stain for pathological parameters including histologic subtype, grade, lymph node metastatic status, and clinical stage. Histologic grades of ovarian cancer included well, moderately, and poorly differentiated. The tissue specimens obtained at surgery were put in liquid nitrogen and stored at –70°C until use.

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2-DE analysis

Ovarian tissue fragments were ground to powder in liquid nitrogen. Sample powder was extracted by PBS buffer containing protease inhibitor. After centrifuge the supernatant was precipitated by adding TCA to final 5%. The pellet was resolved by 8 M urea and 0.1 M DTT. Total 450 µg protein sample was applied to the 2-DE assay, then was rehydrated using Immobiline DryStrip pH 4-7, 13 cm (Amersham Pharmacia Biotech) in strip holder for the first-dimension isoelectric focusing (IEF) overnight at room temperature (RT). IEF was performed using the Multiphor II electrophoresis system. Following rehydration, the strips were focused at: 400V, 1 h; 400V~3500V (gradient) 1.5 h, final 3500V for a total of 70 kVh. Then, strips were equilibrated for 15 min in 1% (w/v) DTT buffer containing 0.05 M Tris-HCl (pH 8.8), 6 M urea, 2% (w/v) SDS, and 30% (v/v) glycerol, and then re-equilibrated for 15 min in the same equilibration buffer containing 2.5% (w/v) iodoacetamide in place of DTT. In the second-dimension separation, the proteins were separated in 12.5% polyacrylamide gel by 20 mA constant current and running buffer containing 0.025 M Tris pH 8.8, 0.192 M glycine and 0.1% SDS, and stained with silver staining [9].

The image from Silver stained gel was scanned with ScanMaker-8700 (Microtek, Hsinchu, Taiwan) and analyzed with the ImageMaster™ 2D Elite (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

MALDI-TOF MS analysis

The spots of interest were cut from the gel and washed with ddH2O before de-staining with 0.025 M ammonium bicarbonate/50% acetonitrile (ACN). The protein in the gel spot was digested overnight by trypsin at 37°C, and the proteolytic peptide fragments were extracted with 1% trifluoroacetic acid (TFA)/50% ACN. After lyophilized, the extracted peptides were dissolved in 30% ACN.

The digests were mixed with α-cyano-4-hydroxycinnamic acid solution (concentration: 50 nmol/µl) in acetonitrile/H_2O and spotted onto a MALDI sample plate. The MALDI-TOF MS analysis was performed on an Autoflex® workstation (Bruker Daltonics, Bremen, Germany) equipped with a 337 nm nitrogen laser. The peptide spectra, acquired in reflectron mode at an accelerating voltage of 20 kV, were the sum of 50 laser shots. The mass spectra were externally calibrated using low mass peptide standards. This procedure typically results in mass accuracies of 50-100 ppm. The de-isotope tryptic peptide fragments were used for protein identification through the Mascot search engine based on the peptide mass fingerprinting of SwissProt protein databases.

SDS-PAGE and Western/Dot blotting analysis

For SDS-PAGE, 30 µg protein was applied to each lane. All samples were heated for 5 min at 95°C before loading to the 15% acrylamide gel. After electrophoresis proteins were electroblotted onto polyvinylidene difluoride (PVDF) membranes. For dot blotting, 5 µg protein was loaded onto PVDF membranes. The membranes were treated with blocking reagent (5% nonfat dried milk, 2% Tween 20, 1 x PBS) for 1 h at RT. The membranes were then probed with anti-retinol binding protein antibody (USBiological, Cat# R1701-16), anti-cathepsin B antibody (USBiological, Cat# C2097-03D), and anti-galectin-1 antibody (Novocastra, Cat# NCL-GAL1) in blocking solution for 2 h at RT. After washing with PBST (0.05% Tween 20, 1 x PBS), the membranes were incubated with horseradish peroxidase-conjugated anti-immunoglobulin antibody in blocking reagent for 1 h at RT. After additional washing with PBST, signals were developed by Western Lightning Chemiluminescence Reagent Plus (PerkinElmer). Signal intensities were scanned with UMAX Astra 4000U. The images were analyzed by GenePix 6.0 for dot blotting, and Fujifilm Science Lab 98 (Image Gauge V3.12) for Western blotting.

Biochemical function and pathway analysis

The PathwayAssist™ software (Strategene, La Jolla, CA, USA) was used to identify functional interrelationships among the protein analyzed in the present study. This software uses the KEGG, DIP and BIND database and natural language scans of Medline to define functional related genes or protein.

Results

A total of 56 tissue specimens were analyzed, which comprised 36 epithelial ovarian cancers and 20 normal ovaries. The clinical and histologic characteristics of the 36 ovarian cancers are summarized in Table 1. There

Table 1. — *Clinical and histologic characteristics of ovarian cancer tissue samples*.

Serial number	Age	Histologic type	Stage	Grade of differentiation*
1	43	Clear cell carcinoma	Ia	3
2	45	Clear cell carcinoma	Ia	3
3	48	Clear cell carcinoma	Ia	3
4	43	Clear cell carcinoma	Ib	3
5	52	Clear cell carcinoma	Ic	3
6	48	Endometrioid adenocarcinoma	Ia	1
7	81	Endometrioid adenocarcinoma	Ia	1
8	46	Endometrioid adenocarcinoma	Ic	1
9	48	Mucinous cystadenocarcinoma	Ia	1
10	36	Mucinous cystadenocarcinoma	Ia	2
11	65	Serous papillary adenoarcinoma	II	2
12	41	Serous cyadenocarcinoma	IIa	3
13	56	Serous adenocarcinoma	IIa	3
14	56	Serous carcinoma	IIa	2
15	70	Serous cystadenocarcinoma	IIb	2
16	59	Endometrioid adenocarcinoma	IIa	1
17	62	Serous cystadenocarcinoma	III	3
18	54	Serous papillary adenoarcinoma	III	3
19	61	Squamous cell carcinoma	IIIa	3
20	58	Clear cell carcinoma	IIIc	3
21	60	Clear cell carcinoma	IIIc	3
22	46	Endometrioid adenocarcinoma	IIIb	1
23	44	Endometrioid adenocarcinoma	IIIc	3
24	56	Serous carcinoma	IIIb	3
25	61	Serous cystadenocarcinoma	IIIc	2
26	70	Serous papillary adenoarcinoma	IIIb	2
27	78	Serous papillary adenoarcinoma	IIIb	3
28	42	Serous papillary adenoarcinoma	IIIc	2
29	70	Serous papillary adenoarcinoma	IIIc	2
30	46	Serous papillary adenoarcinoma	IIIc	2
31	82	Serous papillary adenoarcinoma	IIIc	3
32	72	Serous papillary adenoarcinoma	IIIc	3
33	71	Serous surface papillary adenocarcinoma	IIIb	2
34	59	Serous surface papillary adenocarcinoma	IIIc	3
35	47	Mixed adenocarcinoma (Endometrioid & Serous)	IV	3
36	51	Serous cystadenocarcinoma	IV	3

* Differentiation: Grade 1, well differentiated; Grade 2, moderate differentiation; Grade 3, poor differentiation.

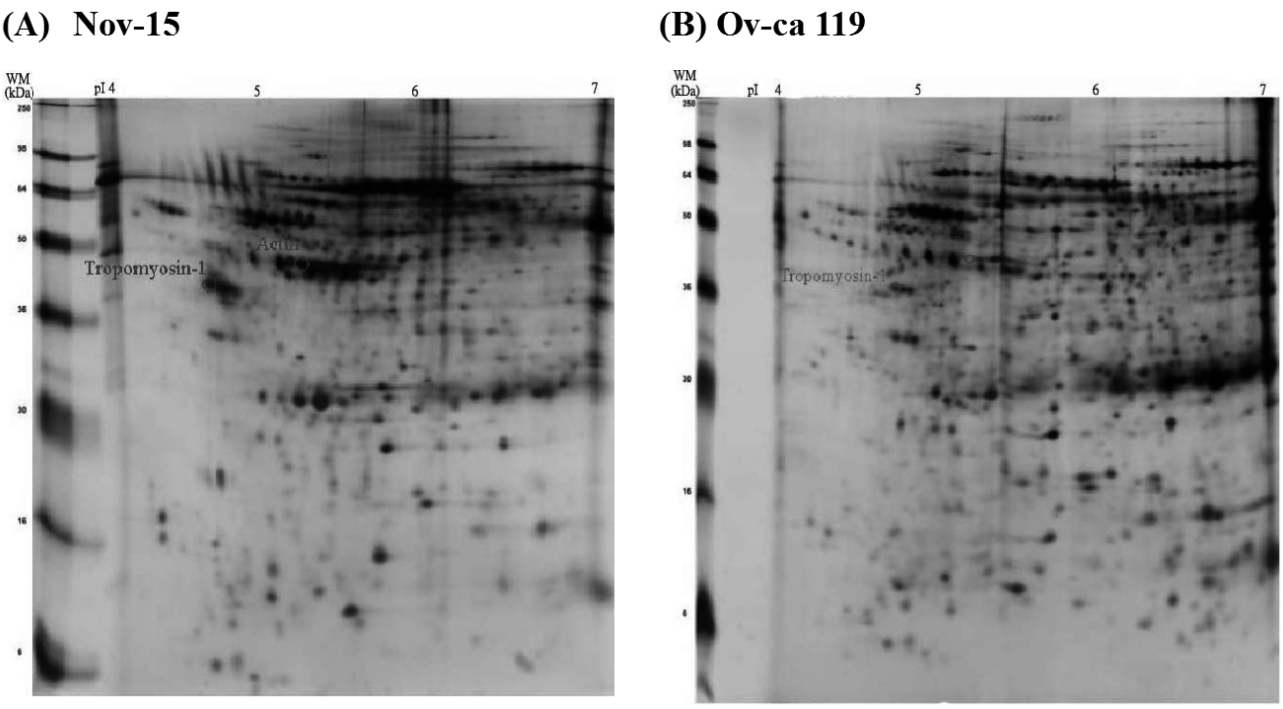

Figure 1. — Representative 2-DE maps.
(A) Protein profile from normal ovarian tissue.
(B) Protein profile from ovarian cancer tissue.

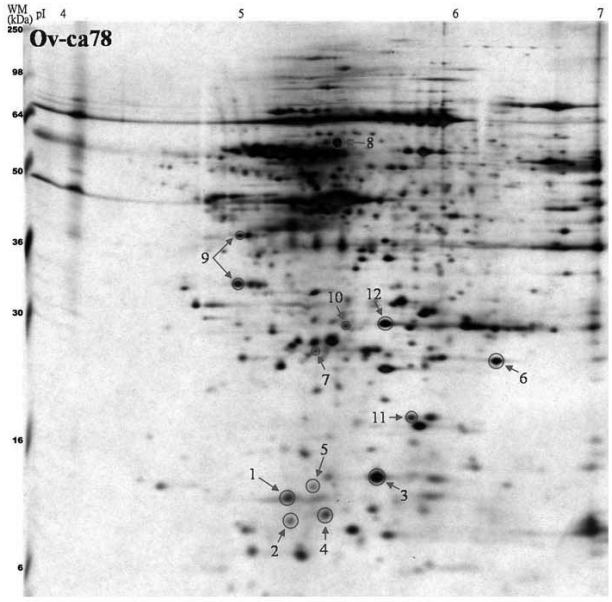

Figure 2. — Locations of several interesting protein spots showing differential expression.
1. CRBP; 2. Gal-1; 3. TTR; 4. SH3BGRL; 5. TCA; 6. DJ-1; 7. CathB; 8. GA; 9. TPM4; 10. UCH-L1; 11. HLA-DRB; 12. HSP27.

were ten cancer tissue samples in Stage I, six in Stage II, 18 in Stage III, and two in Stage IV. Protein extracts from normal ovaries and ovarian cancers were separated on SDS-PAGE before 2-DE. The total amount and quality of protein extracts are further verified by Western blot with mouse anti-actin monoclonal antibody. Representative 2-DE maps from normal and malignant ovarian tissues are shown in Figure 1.

Down- or up-regulated protein expression between normal ovaries and ovarian cancer were evaluated using ImageMaster™ 2D Elite. In comparison with the 2D Elite software quantification, the spots which showed more than a 20% increase or decrease in intensity as compared with a normal ovary were defined as up- or down-regulated spots. According to this definition, more than 30 protein spots showing differential expression were observed in 2-DE maps. Among the protein candidates, 12 protein spots were selected and identified unequivocally. Locations of the 12 interesting protein spots are shown in Figure 2. Characterization of these protein spots is listed in Table 2. Representative expression of protein spots in 2-DE among normal and malignant ovarian tissues are demonstrated in Figure 3. Signal intensities of differential expression of protein spots in 2-DE maps analyzed by ImageMaster™ 2D Elite are shown in Figure 4. In ovarian cancer, up-regulated spots are galectin-1 (Gal-1), cathepsin B (CathB), ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), HLA class II antigen DRB1-11 (HLA-DRB), and heat shock protein 27 (HSP27). Down-regulated spots were cellular retinol-binding protein (CRBP), transthyretin (TTR), SH3 binding glutamic-rich-like protein (SH3BGRL), tubulin-specific chaperone A (TCA), protein DJ-1, gamma-actin (GA) and tropomyosin 4 (TPM4). The differences in signal intensities were statistically significant (p value < 0.05) between cancerous and normal ovaries in the

Table 2. — *Identification of protein spots demonstrating differential expression in 2-DE among normal and malignant ovarian tissues.*

Spot no.	Accession number	Protein description	Score	Coverage (%)	MV / PI*
1	P09455	Cellular retinol-binding protein	577	58	15709/4.99
2	P09382	Galectin-1	157	34	14575/5.34
3	P02766	Transthyretin	854	73	15877/5.52
4	O75368	SH3 binding glutamic-rich-like protein	205	34	12766/5.22
5	O75347	Tubulin-specific chaperone A	264	43	12716/5.25
6	Q99497	Protein DJ-1	447	57	19834/6.33
7	P07858	Cathepsin B	110	13	37797/5.88
8	Q5U032	Gamma-actin	117	5	41766/5.31
9	P67936	Tropomyosin 4	808	59	28373/4.67
10	P09936	Ubiquitin carboxyl-terminal hydrolase L1	416	46	24808/5.33
11	P20039	HLA class II antigen, DRB1-11 beta chain precursor	54	3	30141/6.71
12	P04792	Heat shock protein 27	497	71	22768/5.98

Peptide profiles of the protein spots were analyzed by MALDI-TOF MS and by using the Mascot program.
*The MW (molecular weight)/PI (isoelectric point) of proteins were retrieved from the database of Swiss-Prot/TrEMBL, USA.

Table 3. — *Dot blot analysis of expression of proteins in different stages of ovarian cancer compared with normal ovarian tissues.*

Spot no.	Protein name	Tissue types and stage*		Signal intensity (mean ± SD)	Ratio
1	CRBP	Ov-ca	I+II	4813.14 ± 3298.03	0.38†
			III	10771.54 ± 7667.56	0.84
			I~III	8744.68 ± 6806.33	0.69†
		Normal		12748.84 ± 4763.91	
2	Gal-1	Ov-ca	I+II	10276 ± 4532.66	1.09
			III	15073.8 ± 4235.92	1.60†
			I~III	12941.44 ± 4896.63	1.38†
		Normal		9401 ± 4230.29	
6	DJ-1	Ov-ca	I+II	16127.62 ± 4705.28	0.81†
			III	15510.54 ± 3127.45	0.78†
			I~III	15770.36 ± 3760.55	0.79†
		Normal		19857.90 ± 4073.97	
7	CathB	Ov-ca	I+II	374300.75 ± 108412.76	2.99†
			III	378754.60 ± 125816.21	3.03†
			I~III	376775.11 ± 115001.00	3.01†
		Normal		125072.27 ± 79980.63	

*Stage I + II (n = 8); Stage III (n = 11); Normal ovary (n = 20).
†$p < 0.05$, cancer vs normal (Student's unpaired *t*-test).

expressions of TTR, SH3BGRL, TCA, DJ-1, CathB, GA, TPM4, HLA-DRB and HSP27.

To verify and confirm the signal intensities of protein spots in 2-DE maps, SDS-PAGE and Western/Dot blotting analysis were further performed on four proteins: CRBP, Gal-1, DJ-1 and CathB. Western/Dot blotting analyses of these four proteins are shown in Figure 5 and Table 3, respectively. As shown again in Figure 5 and Table 3, Gal-1 and CathB were up-regulated, whereas CRBP and DJ-1 were down-regulated in ovarian cancer tissues. A similar trend of regulation was also observed in the analyses of 2-DE maps (Figure 4). These results strengthen the validation of 2-DE analysis in this report.

The functional interrelationship networks built by using PathwayAssist™ software between CathB and Gal-1, as well as between CRBP and SH3BGR, are shown in Figures 6 and 7, respectively.

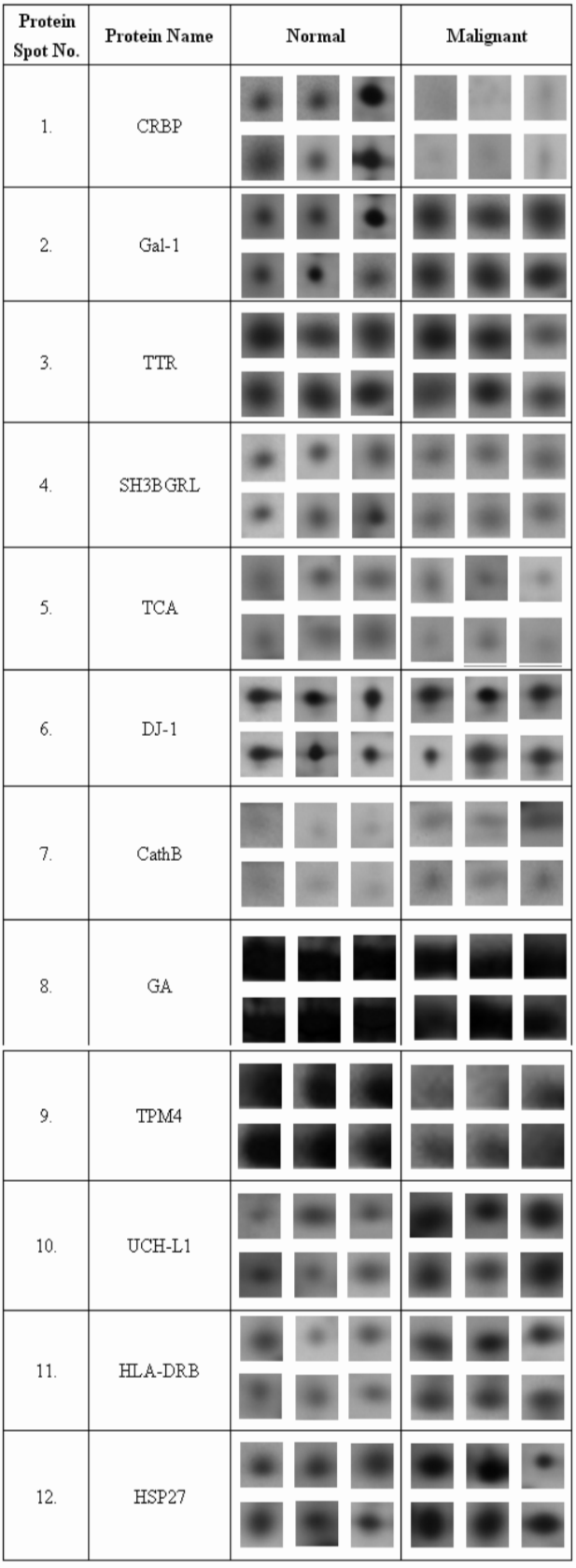

Figure 3. — Representative expression of protein spots in 2-DE among normal and malignant ovarian tissues.

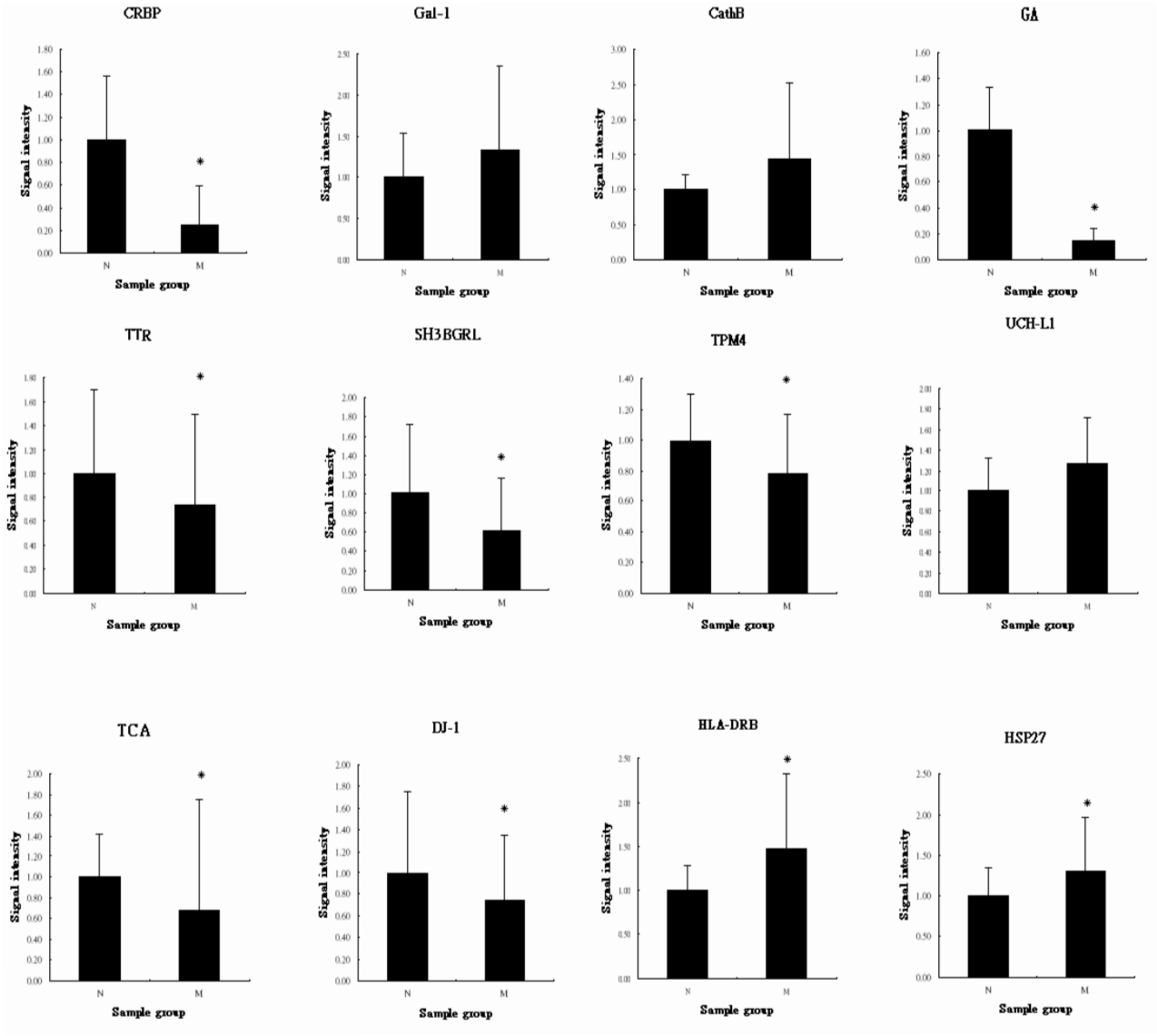

Figure 4. — Histogram of differential expression of protein spots separated in 2-DE maps among normal and malignant ovarian tissues. The mean signal intensity (± S.D.) is shown for each protein.
N: normal; M: malignant.
*: cancer vs normal, $p < 0.05$

Discussion

In this study, we used the tissue specimens of malignant ovarian tumors and normal ovarian tissue to study the protein profiles. Upon comparing the profile, we could get 12 candidate proteins. Among the proteins identified, five proteins (CRBP, Gal-1, TTR, CathB, and HSP27) were previously known proteins involved in tumor progression or differentially expressed in ovarian tumor, while seven proteins (SH3BRL, TCA, DJ-1, GA, TPM4, UCH-L1, and HLA-DRB) were newly identified in our study.

CRBP is essential for vitamin A homeostasis. Cvetkovic *et al*. [10] have reported that there was no detectable CRBP gene expression in 35% of the ovarian cancer samples studied. In addition, down-regulation of the CRBP was also noted in breast [11] and other human cancers [12]. Gal-1, a member of the mammalian beta-galactoside-binding proteins, is involved in several biologic events including regulation of cancer cell proliferation and adhesion to the matrix. Expression of Gal-1 has been documented in many tumor types including the ovary, colon, bladder, melanoma and prostate [13]. TTR plays an important physiologic role in vitamin A homeostasis by its binding to the specific transport protein for retinol [14]. Down-regulation of TTR has been identified in ovarian carcinoma patients using serum proteomic analysis by Zhang *et al*. in 2004 [15]. CathB is a lysosomal cysteine protease whose expression and trafficking are frequently altered in cancers. Nishikawa *et al*. reported that CathB was evident in cancer cells and associated stromal tissues and may contribute to the mechanisms of invasion of ovarian cancer [16]. HSP27 is

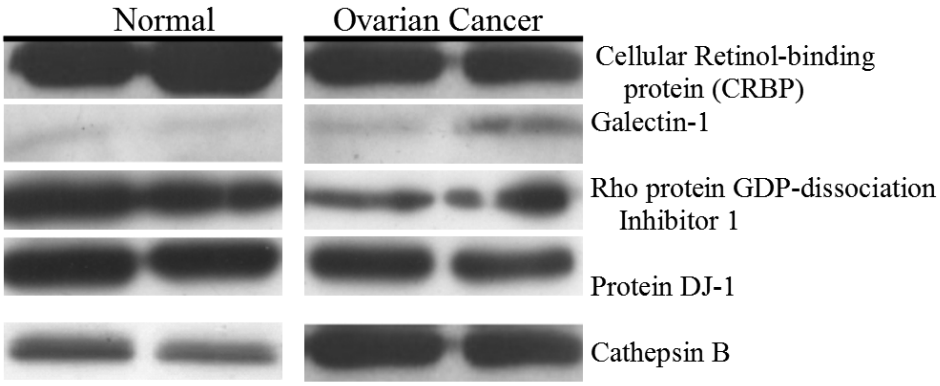

Figure 5. — Western blot analysis of CRBP, Gal-1, DJ-1 and CathB in the tissue homogenate of a normal ovary and ovarian cancer. The expression of Gal-1 and CathB showed up-regulation, but DJ-1 and CRBP showed down-regulation in ovarian cancer tissues.

related to cell growth, tumor invasion, and resistance to chemotherapeutic drugs [17]. In ovarian carcinomas, HSP27 levels are significantly higher in malignant than in benign or borderline tumors, and in Stage II-IV tumors than in Stage I tumors [18]. In addition, there may be a relation between HSP27 expression and worse prognosis in higher stage ovarian carcinoma [19]. All of the above five proteins have been reported to be involved in ovarian tumorigenesis, and our results are consistent with those of previous reports.

SH3BGRL is a member of the SH3BGR family of proteins. The SH3BGR family encodes for a protein that is characterized by the presence of a proline-rich region containing the consensus sequence for a SH3-binding domain and by an acidic carboxyl-terminal region containing a glutamic acid-rich domain. Protein interactions involving SH3-domains have been implicated in signal transduction, cytoskeletal rearrangements, membrane trafficking, and other key cellular processes [20]. Recently, Majid *et al*. [21] reported the down-regulation of SH3BGRL as an important step for v-Rel-mediated transformation. In our study, the SH3BGRL levels were found to be down-regulated in ovarian cancerous samples. The role played by the SH3BGRL protein in ovarian cancer deserves to be elucidated.

The folding pathway of tubulins includes highly specific interactions with a series of cofactors (A, B, C, D and E). Both cofactors A and D function by capturing and stabilizing beta-tubulin in a quasi-native conformation [22]. Our study demonstrated down-regulation of TCA in ovarian cancer. This observation indicates that TCA was critical for the maintenance of microtubules in normal ovarian cells, and suggests that altered function of tubulin cofactors might be implicated in human cancer.

Protein DJ-1 has been suggested to be a novel mitogen-dependent oncogene product involved in a ras-related signal transduction pathway [23]. DJ-1 affects cell survival, in part, by modulating cellular signaling cascades such as PTEN/phosphatidylinositol 3-kinase/Akt [24] and altering p53 activity [25]. The expression of DJ-1 correlates negatively with clinical outcomes in non-small cell lung carcinoma patients [24]. In our study, DJ-1 was significantly down-regulated in ovarian cancer tissue. The discrepancy among the different types of cancers needs further investigation.

Both GA and TPM4 are essential components of cytoskeleton. Actin is the major component of the thin filaments of muscle cells and of the cytoskeletal system of nonmuscle cells. TPM4 is a member of the TPM family. TPM is normally found inside the cell and is associated with the actin cytoskeleton, where it plays a critical role in stabilizing actin filaments in a variety of cell types [26]. Down-regulation of GA and TPM can result in the decrease of cell adhesiveness, hence enhancement of cell motility and metastasis. Previous studies have demonstrated that specific TPM isoforms are down-regulated in human breast carcinoma cell lines [27, 28]. In our study, we found that GA and TPM4 were significantly down-regulated in malignant tissues in comparison to normal ovaries, indicating that GA and TPM4 may play an important role in ovarian cancer metastasis.

UCH-L1 plays an important role in protein degradation through recycling free ubiquitin by cleaving ubiquitylated peptides [29]. Expression of UCH-L1 is limited to neuronal tissue, testes and ovaries [29]. However, up-regulation of UCH-L1 in various tumors including leukemia, medullary thyroid carcinoma, colorectal cancer and breast cancer have been reported, indicating the involvement of UCH-L1 up-regulation in the pathogenesis of these tumors [29-32]. Until now, no report has been available on the expression status of UCH-L1 in ovarian cancer.

The MHC comprises a family of highly polymorphic genes encoding a set of transmembrane proteins that present peptide epitopes to specific antigen receptors on T cells. The HLA system is the human version of the MHC. There are several sorts of hypotheses about the involvement of MHC-antigens in the development of cancer. One hypothesis describes mechanisms by which the tumor itself evades immune surveillance (immune escape) [33]. Workers from our institution recently discovered disruptions of the HLA genotype and down-reg-

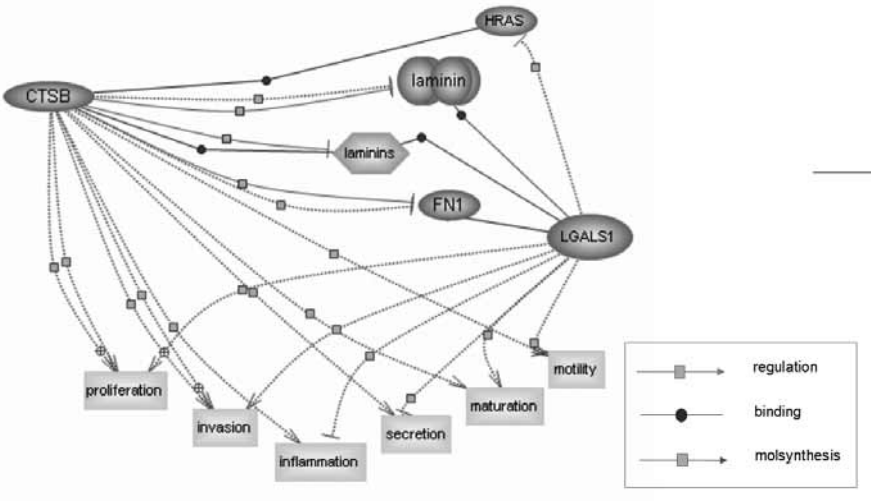

Figure 6. — A pathway built by PathwayAssist™ between CathB and Gal-1. This pathway showed the functional interrelationships of these two proteins. CTSB represents CathB and LGALS1 is the same protein as Gal-1. When using the PathwayAssistTM software program, each connecting line is a "clickable" link that displays the underlying text that supports the interaction.

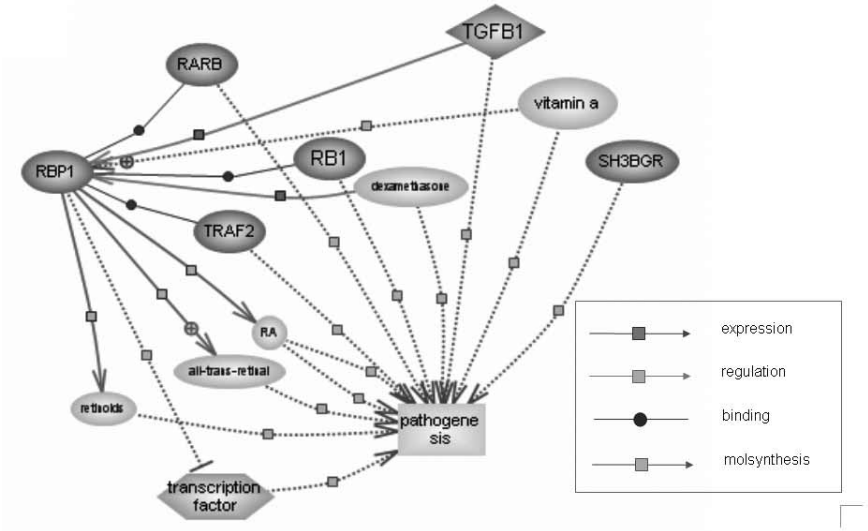

Figure 7. — PathwayAssist™ built pathways between CRBP and SH3BGR proteins. This pathway demonstrates the networks of functional relationships of these two proteins. RBP1 represents CRBP.

ulation of HLA-class I molecules in human cervical carcinoma integrated with high-risk human papillomavirus (HPV) DNA [34]. In that report, the significant mutations of the HLA genotype with reduced HLA-class I molecule expression may possibly be the tactics carried out by HPV to escape the immune attack, thus achieving carcinogenesis. Recently, Kubler *et al*. reported that HLA-class II loci or individual HLA-class II haplotypes might be involved in the pathogenesis of ovarian cancer [35]. In our present study, HLA-DRB expression was significantly higher in cancer tissue compared to normal ovaries (Figure 4). In contrast to the cervical cancer integrated with high-risk HPV DNA, the tumor immune escape mechanism by MHC antigen in ovarian cancer seems to be different. The MHC antigen expression of ovarian cancer is worth further investigation.

The functional interrelationship network of several interested proteins among the 12 protein profiles studied in this report was built by using PathwayAssist™ software. As illustrated in Figure 6, CathB and Gal-1 are functionally related to proliferation, invasion and motility of cancer cells, and both were found to be up-regulated in our study. Figure 7 demonstrates the functional network between CRBP and SH3BGR protein; these two proteins play important role in cancer pathogenesis through many pathways.

In conclusion, we have demonstrated in the present study that 12 protein spots are expressed differentially on ovarian cancers and normal ovaries, and seven identified proteins have not previously been reported in ovarian cancer. Although we could not completely explain all correlations between expressed proteins and their roles in

tumorigenesis, there might be a possibility to find tumor-specific markers among the differentially expressed proteins. Further cloning and functional analysis of these proteins will provide more information on pathophysiologic roles during tumor formation and progression.

Acknowledgements

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