Comparative proteomic analysis of the esophageal squamous carcinoma cell line EC109 and its multi-drug resistant subline EC109/CDDP

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Abstract. To gain insights into the mechanisms of drug resistance in esophageal squamous cell carcinoma (ESCC), we employed proteomic techniques to study the global protein change of the multi-drug resistant ESCC cell line EC109/CDDP, which was established in our previous work, in comparison with its parental drug sensitive cell line EC109. By two-dimensional electrophoresis and mass spectrometry, we successfully identified 44 proteins with altered expression levels. These proteins are involved in endoplasmic reticulum stress response, metabolic process, DNA replication and repair, nucleotide binding, calcium binding, and cytoskeletal proteins. Among them, the differential expression levels of thioredoxin domain-containing protein 4 precursor and cystathionine γ-lyase were further validated by Western blot and RT-PCR. Our present results lay foundation for future in-depth work on molecular mechanism of ESCC drug resistance, and aid in the identification and use of novel markers in clinical practice.

Introduction

Esophageal cancer (EC) is one of the most common malignancies in the world. It was estimated that >300,000 people die of it every year, ranking as the sixth leading cause of cancer death worldwide (1). Most esophageal cancers in China are squamous cell carcinomas (SCCs), which are different from the most prevalent esophageal cancers, adenocarcinomas, in Western countries, leading to distinct etiologies, therapeutics and prognosis (2). Despite advances in surgical therapy for esophageal squamous cell carcinoma (ESCC), the overall prognosis of patients has not improved markedly during the past few decades due to the fact that most patients have locally-advanced or disseminated diseases at diagnosis (3). For these unresectable diseases, chemotherapy alone (stage IV) or in combination with other local treatment modalities (stages II and III) would be an optimal choice. A recent meta-analysis indicated that preoperative chemoradiotherapy with surgery would bring about 13% absolute difference in survival at 2 years versus surgery alone (4). However, the success of chemotherapy depends on the sensitivity of the tumor to the antineoplastic agents. ESCC cells often acquire resistance to drugs and even develop multiple drug resistance, which results in treatment failure. To achieve more effective chemotherapeutic treatment of ESCC patients, it is essential to define reliable indicators of response to treatment in individual patients and to make clear which mechanisms are responsible for drug resistance.

A lot of work has been done on the molecular mechanisms of ESCC drug resistance. It has been reported that factors including growth-factor receptor, angiogenetic factors, tumor suppressor genes, cell cycle regulators, and DNA repair enzymes are potential EC chemo-sensitivity predictive markers (5-11). The development of techniques within genomics and proteomics enables extensive characterization of malignant tumors, which may help in understanding treatment resistance and/or treatment sensitivity on the global scale. Gene expression studies on mRNA level have shown to be able to detect molecular signature linked to acquired resistance to cisplatin in ESCC cells (12). However, several aspects in tumor biology cannot be captured by gene expression analysis only, such as protein expression levels, protein degradation and post-translational modifications, emphasizing the need for complementary analysis at the protein level. Proteomic studies based on two-dimensional electrophoresis (2-DE) have been adopted to study chemotherapy resistance of various tumor cell lines, including breast cancer, lung cancer, and colon cancer (13). However, there is no previous global protein analysis by 2-DE on ESCC cell lines of different drug sensitivity. We still lack protein markers that show direct relevance to ESCC chemoresistance.

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To gain new insights into the mechanisms of drug resistance in ESCC, we employed proteomic techniques to analyze protein extracts from the ESCC drug resistant cells EC109/CDDP (14) and its parental cells EC109 in the present study. After comparing the expression patterns, differentially expressed proteins were identified by mass spectrometry (MS) and further validated by Western blot and RT-PCR.

Materials and methods

Cell culture. Both EC109 and EC109/CDDP cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, NY, USA) supplemented with 10% fetal bovine serum, 100 U/ml penicillin (Tianxin, Guangzhou, China) and 100 U/ml streptomycin (Merro, Dalian, China) in a humidified incubator containing 5% CO_2 at 37˚C. The EC109/CDDP cells were generated by exposing EC109 cells to cisplatin at 2 h pulse treatment of 25 μM for 6 times (14). Previous studies identified that EC109/CDDP cells, as compared with the parental EC109 cells, showed cross-resistance to cisplatin, carboplatin, 5-fluorouracil, taxol, navelbine, irinotecan and etoposide. Only EC109 and EC109/CDDP cells with the IC_{50} of cisplatin at ~1.0 μg/ml and 8.5 μg/ml determined by MTT assay (14), respectively, were used for the following 2-DE, Western blot and RT-PCR analyses, in order to guarantee the homogeneity of the cells collected, at least, regarding their resistance to cisplatin.

Two-dimensional gel electrophoresis. EC109/CDDP cells and EC109 cells were harvested and dissolved in proper amount of lysis buffer containing 7 M urea (Amersham Biosciences, Piscataway, NJ, USA), 2 M thiourea (Amersham Biosciences), 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Amersham Biosciences), 40 mM 1,4-dithio-DL-threitol (DTT) (Amersham Biosciences) and a protease inhibitor cocktail tablet (Roche Applied Science, Mannheim, Germany). The protein concentration was determined by 2-D Quant Kit (Amersham Biosciences) and stored at -80˚C.

The 2-DEs were carried out as described (15). Each precast immobilized pH gradient (IPG) strip (pH 3-10 NL, 18 cm) (Amersham Biosciences) was loaded with 50 μg proteins diluted in 340 μl rehydration solution containing 7 M urea, 2 M thiourea, 4% CHAPS, 40 mM DTT, 0.5% IPG buffer (pH 3-10, NL) (Amersham Biosciences) and a trace of bromophenol blue. Rehydration and IEF were performed in a Protean IEF cell apparatus (Bio-Rad, Hercules, CA, USA) using following conditions: (1) 50 V, 12 h; (2) 250 V, 15 min; (3) 10,000 V, 3 h, linear; (4) 10,000 V, 50,000 VH. Then, the strips were equilibrated in the SDS equilibration buffer (6 M urea, 75 mM Tris-HCl pH 8.8, 29.3% glycerol, 2% SDS and 0.002% bromophenol blue) containing 1% DTT for 15 min and then in another SDS equilibration buffer containing 2.5% iodoacetamide instead of DTT for additional 15 min. For the second dimension, 12.5% SDS-PAGE were run in Protean II XL cell apparatus (Bio-Rad). For each sample, three replicate gels were run.

After electrophoresis, the protein gels were visualized by silver stain using PlusOne Silver Staining Kit (Amersham Biosciences) according to the manufacturer's instruction. The method was modified to be compatible with mass spectrometry analysis by omitting glutaraldehyde from the sensitizer solution.

Analysis of gel images. Silver-stained gels were scanned using a Molecular Imager GS-800 Calibrated Densitometer (Bio-Rad). Raw images from 2-DE gels were analyzed using the PDQuest 2-D Analysis Software (Version 7.4, Bio-Rad) according to the manual. The raw quantity of each spot in a gel was normalized by the total quantity of valid spots in that gel in order to accurately compare spot quantities between gels. The three replicate gels of each sample were defined as a replicate group. The spots displaying a change in their expression between the two groups greater than the two-fold factor (determined from the mean) significantly ($P<0.05$) were considered as differentially expressed proteins.

Mass spectrometric analysis of proteins. Silver-stained protein spots of interest were excised from the gels and digested with trypsin (20 μg/ml, Promega Corporation, Madison, WI, USA) according to manufacturer's instruction. Then the samples were analyzed in a positive ion reflector mode on a 4800 plus MALDI-TOF-TOF Analyzer Proteomic Analyzer (Applied Biosystems, Foster City, CA, USA). Five MS peaks of each sample with a signal-to-noise ratio above 50 were subjected to further MS/MS analysis. All MS and MS/MS data of each individual spot were analyzed by GPS Explore (Version 3.6, Applied Biosystems) and searched in International Protein Index (IPI)_human database searching through MASCOT (Version 2.1, Matrix Science, London, UK). Only identified proteins with a confidence interval (CI) of either protein or ion score >95% were accepted.

Western blot. EC109 and EC109/CDDP cells were dissolved using 1xRIPA lysis buffer (Upstate, Lake Placid, NY, USA), respectively. Proteins were separated using 12% SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Amersham Biosciences). After blocked in Tris-buffered saline containing 5% (w/v) non-fat dry milk and 0.1% (v/v) Tween-20 for 1 h at room temperature, the membranes were then probed with following primary antibodies: rabbit polyclonal anti-thioredoxin domain-containing protein 4 (TXNDC4) antibody (Cell Signaling Technology, Beverly, MA, USA), mouse monoclonal anti-cystathionine γ-lyase (CTH) antibody (Abnova, Jhouzih St., Taibei, Taiwan), and mouse monoclonal anti-ß-actin antibody (Thermo Scientific, Fremont, CA, USA) overnight at 4˚C, followed by incubation with HRP-conjugated secondary antibodies, sheep anti-mouse IgG (Amersham Biosciences) or goat anti-rabbit IgG (Zymed, San Francisco, CA, USA) for 1 h at room temperature. Finally, the protein bands were visualized on the X-ray sensitive film using the enhanced chemiluminescence detection system (Amersham Biosciences). The specific intensity of each protein band on X-ray film was measured by Quantity One V4.62 (Bio-Rad) and expressed as a ratio of the optical density band of each protein to that of ß-actin.

Semi-quantitative RT-PCR. Total RNA was isolated from EC109 and EC109/CDDP cells using TRIzol reagent

Table I. Primers used for RT-PCR analysis.

Gene	Primers (5'_3')	Accession number	Annealing temperature (˚C)	Size (bp)
CTH 1		NM_001902	51	800
Sense	CCATCTCACTGTCCACCAC			
Anti-sense	CTGACGCTTCACCAACTC			
CTH 2		NM_153742	51	668
Sense	CCATCTCACTGTCCACCAC			
Anti-sense	CTGACGCTTCACCAACTC			
TXNDC4		NM_015051	50	240
Sense	TTCCGATGTCATTAAGGAAG			
Anti-sense	GCTAAGTCCCGAATTTCTTG			
ß-actin		NM_001101	55	619
Sense	CGGGACCTGACTGACTACCTCATCAAGA			
Anti-sense	TCAAGAAAGGGTGTAACGCAACTA			

(Invitrogen). RNA was reverse-transcripbed using the First Strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Then cDNA was amplified by PCR using rTaq DNA polymerase (Toyobo). The primers sequences, length of products, and PCR reaction conditions are listed in Table I. Agarose gels (1.5%) were used to separate PCR products, and visualized by Gel Doc (Bio-Rad). The level of specific mRNA was measured by Quantity One V4.62 (Bio-Rad) and expressed as a ratio of the optical density band of each gene to that of the house-keeping gene, ß-actin.

Statistical analysis. Data are expressed as mean ± standard deviation (SD) of at least 3 repeated experiments. Data analysis was carried out utilizing the SPSS 16.0 statistical software package (SPSS, IL, USA). Continuous variables were analyzed using Student's t-test. $P<0.05$ was considered statistically significant, and the reported P-values were two-sided.

Results

Proteomic analysis of EC109 and EC109/CDDP cells. Proteomic profiles of EC109 and EC109/CDDP cells were investigated by 2-DE. Totally 1248 protein spots were observed, which were localized in the range of pI 3-10 with a relative molecular mass of 10-200 kDa. The analysis of the six 2-DE gels obtained for EC109 and EC109/CDDP cells with three replicas per sample showed that 88.7% (1107/1248) and 84.6% (1053/1248) of the spots matched among gels of EC109 and EC109/CDDP cell protein sample, respectively (Fig. 1). The mean overall coefficients of variation (CVs) (SD/mean x100%) of the matched spot quantities in EC109 and EC109/CDDP's replicate gels were 27.6 and 30.6%, respectively, consistent with the conclusion drawn from previous reports that the average baseline technical variation from the 2-DE separation and detection process accounts for quantitative variation in the range of 20-30% (16). Furthermore, 27 spots were found more than two-fold up-regulated and 34 down-regulated in EC109/CDDP cells as compared with EC109 cells by PDQuest analysis ($P<0.05$).

Protein identification. Among the 61 differentially expressed spots between EC109 and EC109/CDDP cells, 55 were cut from the gels for MS analysis and 44 (83.6%) spots were identified with CI of either protein or ion score >95% (Table II; Figs. 2 and 3). Six spots up-regulated in EC109 cells were not subjected to MS analysis, for they were too obscure or faint to cut from gels by visual inspection. Three spots up-regulated in EC109 and 8 in EC109/CDDP cells, respectively, analyzed by MS and MS/MS analysis with the CI of either protein or ion score <95% were not recorded as successfully identified.

Spot SSP 7117 was identified as two unique proteins. Likewise, a large number of spots were identified as proteins with isoforms or variants of one another, such as spots SSP 2117, 6115, 6305, 8103, and 8115. This might be caused by the partial same amino acid sequence of different proteins or isoforms, which could not be distinguished by MS and MS/MS analysis precisely. Further experiments should be performed to confirm which protein or isoform affects the drug-resistance phenotype.

Some of the identified proteins migrated at PI and MW values different to the theoretical ones. Two spots SSP 0308 and 0309, both up-regulated in EC109 cells, were identified as the same protein reticulocalbin-1 precursor (RCN1, IPI00015842). It might be due to either processing or post-translational modification resulting in different positions in the gel for a given gene product. Characterization of these proteins in terms of modification type was not attempted in this study.

Western blot. The protein expression of two identified proteins up-regulated in EC109/CDDP cells of interest by 2-DE and MS, TXNDC4 (SSP 2338) and CTH (SSP 6305), were selected for further validation using Western blot. The

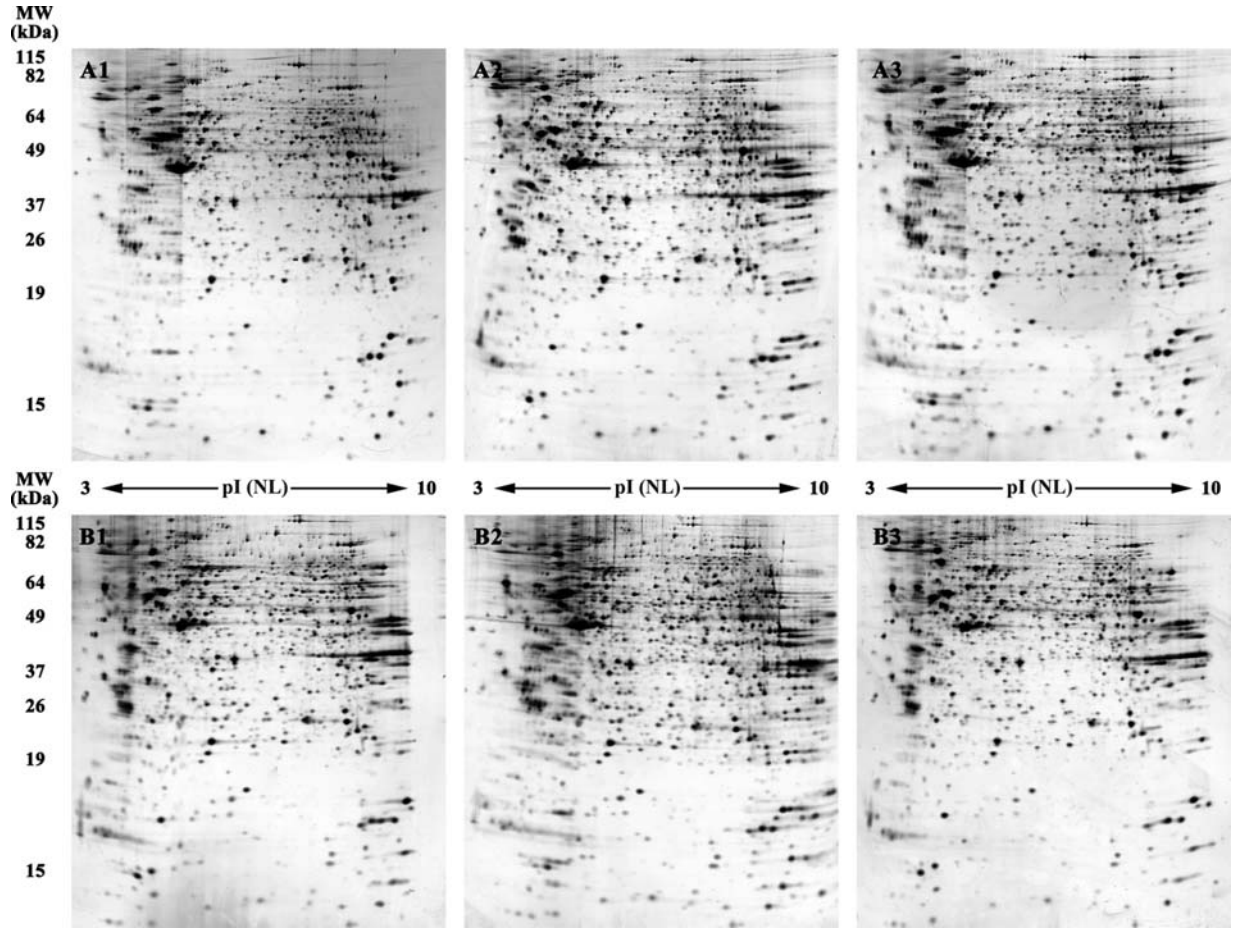

Figure 1. Three replicate 2-DE silver-stained gel images of EC109 (A1-A3) and EC109/CDDP (B1-B3) cell proteins, respectively.

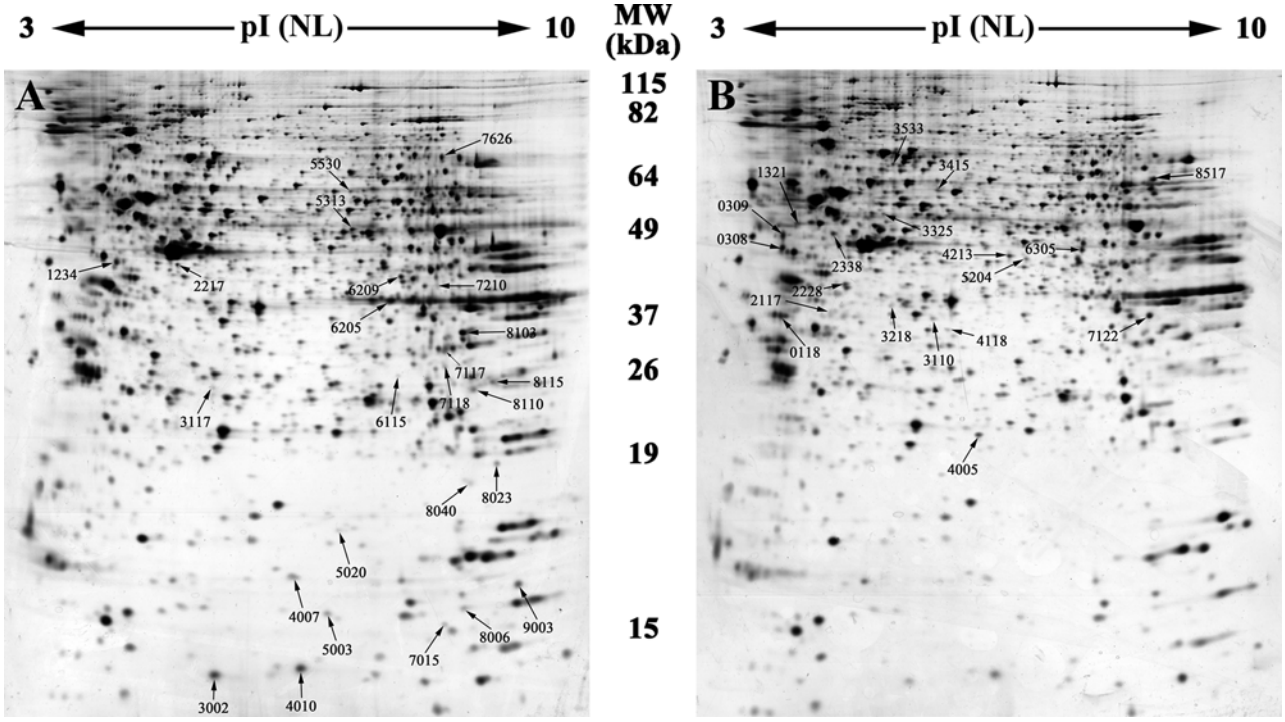

Figure 2. Representative 2-DE gels (pI 3-10, NL, 18 cm) showing the location of 25 protein spots up-regulated in EC109 (A) cells and 19 in EC109/CDDP (B) with arrows. The number in the image is the SSP (standard spot) number assigned uniquely to each spot by PDQuest software.

Table II. Identified increased and decreased proteins in drug-resistant cell line EC109/CDDP relative to parental EC109.

SSP No.	Gene symbol	Protein name	Accession No.[a]	Fold change[b]	Theoretical MW (Da)/pI	Function
0118	TPM1	Isoform 2 of tropomyosin α-1 chain Tropomyosin 1 α variant 6	IPI00745267 IPI00384369	3.24↑[b]	26664/4.77 28720/4.75	Cytoskeletal proteins
0308	RCN1	Reticulocalbin-1 precursor	IPI00015842	4.20↑	38866/4.86	Calcium binding
0309	RCN1	Reticulocalbin-1 precursor	IPI00015842	3.32↑	38866/4.86	Calcium binding
1234	RPSA	40S ribosomal protein SA Ribosomal protein SA	IPI00553164 IPI00413108	2.90↓[b]	32833/4.79 33293/4.79	Protein biosynthesis
1321	VIM	Vimentin	IPI00418471	4.64↑	53619/5.06	Cytoskeletal proteins
2117	SFRS1	Isoform ASF-1 of splicing factor, arginine/serine-rich 1 Isoform ASF-3 of splicing factor, arginine/serine-rich 1	IPI00215884 IPI00218592	9.73↑	27728/10.37 22446/7.72	RNA splicing
2217	SERPINB6	Serine (or cysteine) proteinase inhibitor, clade B (ovalbumin), member 6	IPI00749398	4.15↓	42594/5.18	Protein degradation
2228	EIF2S1	Eukaryotic translation initiation factor 2 subunit 1	IPI00219678	3.25↑	36089/5.02	Protein biosynthesis
2338	TXNDC4	Thioredoxin domain-containing protein 4 precursor	IPI00401264	2.74↑	46941/5.09	Cell redox homeostasis/chaperone
3002	S100A4	Protein S100-A4	IPI00032313	4.57↓	11721/5.85	Calcium binding
3110	STARD3	START domain-containing protein 3 35 kDa protein	IPI00791229	2.83↑	35037/6.04	Metabolism
3117	PSPH	Phosphoserine phosphatase	IPI00019178	2.43↓	24992/5.53	Metabolism
3218	TUBB TUBB2C	Tubulin ß chain Tubulin, ß polypeptide Tubulin ß-2C chain	IPI00011654 IPI00645452 IPI00007752	184.06↑	49639/4.78 47736/4.70 49799/4.79	Cytoskeletal proteins
3325	POLD2	DNA polymerase subunit δ-2	IPI00025616	2.19↑	51257/5.35	DNA replication and repair
3415	PDIA3	Protein disulfide-isomerase A3 precursor	IPI00025252	2.09↑	56747/5.98	Cell redox homeostasis
3533	KRT1	Keratin, type II cytoskeletal 1	IPI00220327	2.48↑	65978/8.16	Cytoskeletal proteins
4005	ABHD14B	ABHD14B 22 kDa protein Isoform 1 of abhydrolase domain-containing protein 14B	IPI00747859 IPI00063827	2.17↑	22367/6.04 22332/5.94	Metabolism
4007	UBE2N	ubiquitin-conjugating enzyme E2N 16 kDa protein	IPI00874051	2.26↓	16233/5.41	DNA repair
4010	S100A11	Protein S100-A11	IPI00013895	3.75↓	11733/6.56	Calcium binding
4118	DNAJC9	DNAJ homolog subfamily C member 9	IPI00154975	2.91↑	29891/5.58	Chaperone
4213	SERPINB1	Leukocyte elastase inhibitor	IPI00027444	2.32↑	42715/5.26	Protein degradation
5003	FABP3	Fatty acid-binding protein, heart	IPI00219684	2.95↓	14849/6.29	Metabolism
5020	ACP1	Isoform 1 of low molecular weight phosphotyrosine protein phosphatase	IPI00219861	2.27↓	18031/7.63	Metabolism
5204	DNAJB11	DnaJ homolog subfamily B member 11 precursor	IPI00008454	2.38↑	40489/5.81	Chaperone

Table II. Continued.

SSP No.	Gene symbol	Protein name	Accession No.[a]	Fold change[b]	Theoretical MW (Da)/pI	Function
5313	ENO1	α-enolase	IPI00465248	2.56↓	47139/7.01	Metabolism/nucleotide binding
	ENO3	ß-enolase	IPI00218474		46957/7.59	Metabolism
5530	TXNRD1	TXNRD1 55 kDa protein	IPI00871867	2.04↓	54512/6.07	Cell redox homeostasis
		Thioredoxin reductase 1, cytoplasmic precursor	IPI00554786		54672/6.07	
		Thioredoxin reductase 1 isoform 1	IPI00847482		60627/6.42	
6115	PSMD9	Isoform p27-L of 26S proteasome non-ATPase regulatory subunit 9	IPI00010860	3.71↓	24639/6.46	Protein degration
		Isoform p27-S of 26S proteasome non-ATPase regulatory subunit 9	IPI00216220		22722/5.38	
6205	ANXA1	Annexin A1	IPI00218918	2.03↓	38690/6.57	Calcium binding
6209	ACAT2	Acetyl-CoA acetyltransferase, cytosolic	IPI00291419	2.14↓	41324/6.47	Metabolism
6305	CTH	Isoform 1 of cystathionine γ-lyase	IPI00031557	2.96↑	44480/6.21	Metabolism
		Isoform 2 of cystathionine γ-lyase	IPI00221301		39480/6.43	
7015	EIF1B	Eukaryotic translation initiation factor 1b	IPI00031489	9.23↓	12816/6.82	Protein biosynthesis
7117	ISOC1	Isochorismatase domain-containing protein 1	IPI00304082	2.54↓	32216/6.96	Unkonwn
7118	PROSC	Proline synthetase co-transcribed bacterial homolog protein	IPI00016346	2.39↓	30325/7.09	Unknown
7122	GAPDH	GAPDH 32 kDa protein	IPI00795257	4.36↑	31528/7.15	Metabolism
		Glyceraldehyde-3-phosphate dehydrogenase	IPI00219018		36030/8.57	
		Glyceraldehyde 3-phosphate dehydrogenase	IPI00789134		27853/6.45	
7210	PCBP2	PCBP2 protein	IPI00470509	2.09↓	35324/8.17	Nucleotide binding
		Poly(rC)-binding protein 2	IPI00216689		38556/8.17	
	PCBP3	Isoform 4 of Poly(rC)-binding protein 3	IPI00410587		35788/8.22	Nucleotide binding
7626	FUBP1	Isoform 2 of far upstream element-binding protein 1	IPI00853059	2.35↓	68691/6.62	Nucleotide binding
		FUBP1 68 kDa protein	IPI00873767		67648/6.85	
8006	PFN1	Profilin-1	IPI00216691	2.67↓	15045/8.44	Cytoskeletal proteins
8023	CNBP	Cellular nucleic acid-binding protein ß variant 2	IPI00430813	8.51↓	18957/7.76	Nucleotide binding
		CNBP zinc finger protein	IPI00430812		19579/7.76	
8040	UBE2M	NEDD8-conjugating enzyme Ubc12	IPI00022597	2.83↓	20887/7.57	Protein degradation
	UBE2MP1	Similar to NEDD8-conjugating enzyme Ubc12	IPI00457179		19091/7.55	
8103	VDAC2	Isoform 3 of voltage-dependent anion-selective channel protein 2	IPI00216026	2.08↓	31547/7.49	Transmembrane proteins
		VDAC2 30 kDa protein	IPI00855744		30329/8.00	
		Isoform 2 of voltage-dependent anion-selective channel protein 2	IPI00216024		30393/6.81	
8110	EIF4H	Isoform long of eukaryotic translation initiation factor 4H	IPI00014263	3.44↓	27368/6.67	Protein biosynthesis

Table II. Continued.

SSP No.	Gene symbol	Protein name	Accession No.[a]	Fold change[b]	Theoretical MW (Da)/pI	Function
8115	AK2	Isoform 2 of adenylate kinase isoenzyme 2, mitochondrial	IPI00218988	2.46↓	25598/7.71	Metabolism/apoptosis
		Isoform 1 of adenylate kinase isoenzyme 2, mitochondrial	IPI00215901		26461/7.67	
		Isoform 3 of adenylate kinase isoenzyme 2, mitochondrial	IPI00172460		22251/8.94	
8517	DAK	Dihydroxyacetone kinase	IPI00551024	2.39↑	58940/7.12	Metabolism
9003	SSBP1	Single-stranded DNA-binding protein, mitochondrial precursor	IPI00029744	2.03↓	17249/9.59	DNA repair

[a]IPI_human database accession number. [b]Fold changes are represented as mean values calculated from three-repeated experiments. ↑, spot quantity increased in EC109/CDDP in comparison to EC109; ↓, spot quantity decreased in EC109/CDDP in comparison to EC109 (Student's t-test, P<0.05).

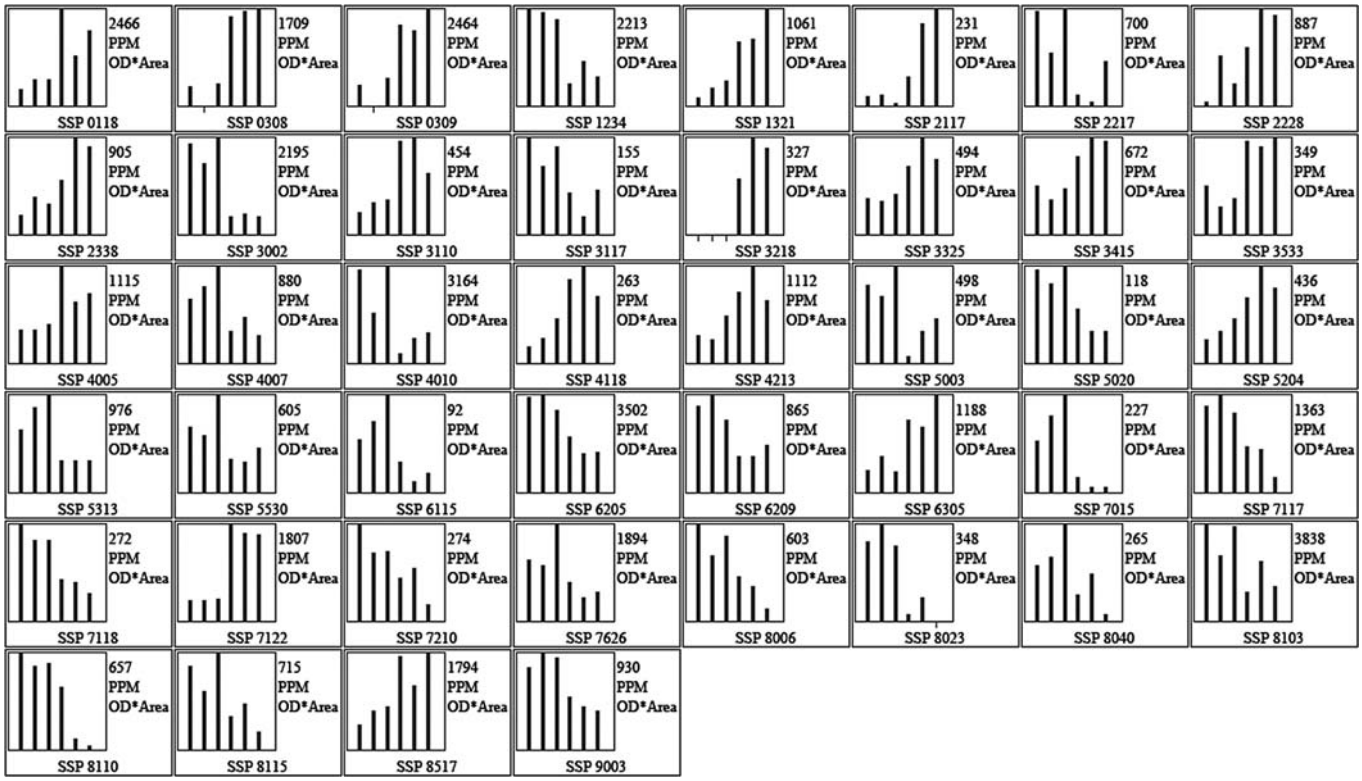

Figure 3. Histogram graphs showing the quantities of 44 differential protein spots between EC109 and EC109/CDDP cells. Bar, protein spot's quantity in each gel. Left three bars, quantities of the protein spots on three separate gels from the parental EC109 cells; right three bars, quantities of the protein spots on three separate gels from the drug-resistant EC109/CDDP cells. SSP, standard spot. The number at the upper right of the histogram is the quantitation of the maximum bar in the graph. The other bars are drawn proportional to the highest bar. PPM (parts per million) is a constant, by which the normalized quantity is multiplied to give a more meaningful value.

Western blot image (Fig. 4A) of CTH showed two distinct bands at approximate 44.5 and 37.5 kDa, respectively, identical to the molecular weights of the two isoforms CTH1 and CTH2. CTH2 protein expression was quite weak, and no marked change was observed between EC109 and EC109/CDDP cells. After normalization to ß-actin, the expression levels of TXNDC4 in EC109 and EC109/CDDP cells were 0.1866±0.04248 and 0.3970±0.08417, respectively, and that of CTH1 was 0.5464±0.02129 and 1.288±0.3903, respectively. The TXNDC4 and CTH1 protein expression ratio of EC109/CDDP to EC109 cells were 2.929 (P<0.001) and 3.244 (P=0.018), respectively (Fig. 4B), consistent with the data shown in Fig. 3 and Table II obtained using the proteomic approach.

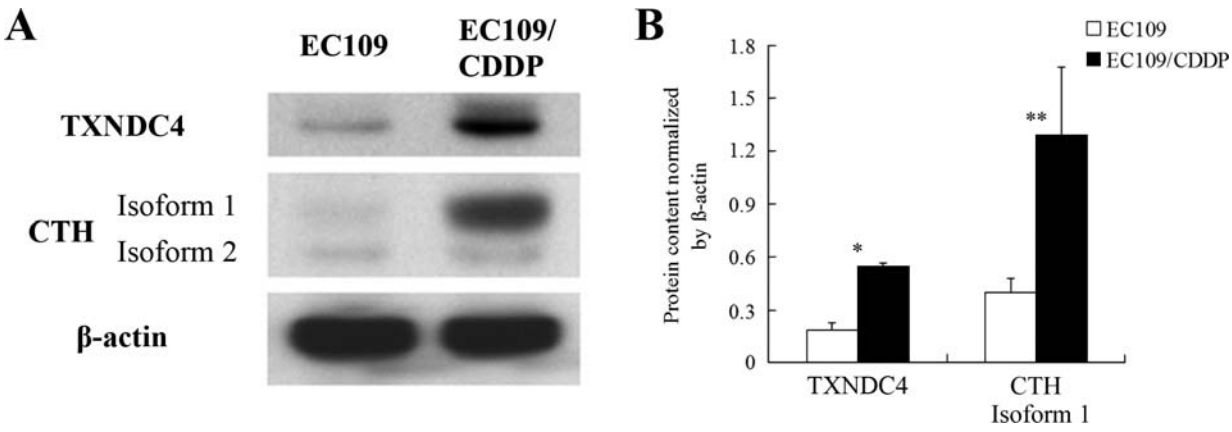

Figure 4. Differential expressed proteins TXNDC4 and CTH identified by proteomic approach were validated by Western blot. (A) Western blot image of TXNDC4, CTH and ß-actin in EC109 and EC109/CDDP cells. (B) Protein expression levels of TXNDC4 and CTH after normalization relative to ß-actin. *P<0.001; **P=0.018.

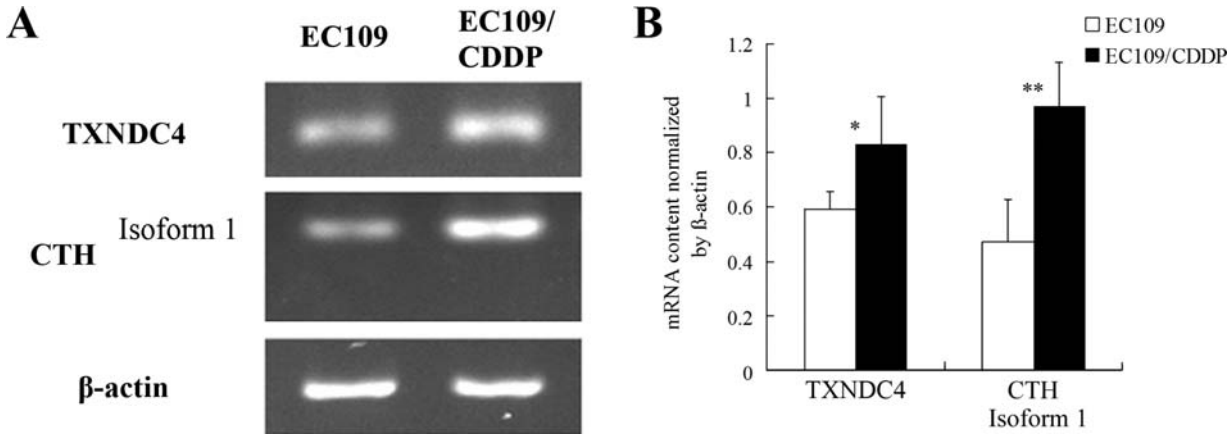

Figure 5. Differential expression of proteins TXNDC4 and CTH identified by proteomic approach were validated by RT-PCR. (A) Agrose electrophoresis image of TXNDC4, CTH and ß-actin PCR products in EC109 and EC109/CDDP cells. (B) mRNA expression levels of TXNDC4 and CTH after normalization relative to ß-actin. *P=0.094; **P=0.018.

Semi-quantative RT-PCR. The mRNA expression of TXNDC4 and CTH in EC109 and EC109/CDDP cells were determined using semi-quantitative RT-PCR. Gene expression profile of TXNDC4 and CTH is shown in Fig. 5A. For CTH, only one band at approximate 800 bp was visible on the gel, which was CTH Isoform 1 PCR product. No CTH Isoform 2 PCR product at 668 bp could be detected in either EC109 or EC109/CDDP cells. After normalized to ß-actin, the expression level of TXNDC4 in EC109 and EC109/CDDP cells was 0.5902±0.06689 and 0.8297±0.1778, respectively, and that of CTH1 was 0.4732±0.1504 and 0.9699±0.1643, respectively. The TXNDC4 and CTH1 mRNA expression ratio of EC109/CDDP to EC109 cells was 1.406 (P=0.094) and 2.050 (P=0.018), respectively (Fig. 5B). The mRNA levels of TXNDC4 and CTH1 in EC109 and EC109/CDDP cells were in accordance with the protein levels based on both 2-DE and Western blot analyses.

Discussion

ESCC patients generally show different degrees of chemosensitivity, even those with the same disease stage and therapeutic regimen. Drug-resistance acts as a barrier in esophageal cancer therapy, and it affects the prognosis of patients in neoadjuvant and adjuvant settings (17,18). Therefore, it is important to elucidate the mechanisms of drug resistance in order to improve the prognosis of esophageal cancer patients.

In our previous work, we established a drug-resistant cell line, EC109/CDDP cells, by pulse treatment of the most widely-used antitumor drug, cisplatin to the ESCC EC109 cells, and carried out experiments intending to find out the potential molecular mechanisms involved in ESCC drug-resistant phenotype. However, the well-known multidrug resistance-related genes, ATP-binding cassette, sub-family B, member 1 (MDR1), ATP-binding cassette, sub-family C, member 1 (MRP1), ATP-binding cassette, sub-family G, member 2 (ABCG2), lung resistance protein (LRP), and glutathione S-transferase π (GST-π) might not completely explain its multi-drug resistance (14). We, therefore, used high throughput proteomics approach to investigate the protein expression change of EC109/CDDP, so as to help understanding of the molecular mechanism of ESCC drug resistance and to identify candidate biomarkers of anticancer drug response or novel therapeutic targets.

Forty-four proteins exhibited at least 2-fold changes of expression level in the multidrug-resistant cell line EC109/CDDP compared with its counterpart EC109 identified by MS analysis. To confirm the proteomic results, two proteins CTH and TXNDC4, in which we are interested, were selected and analyzed by Western blot and RT-PCR. The changes of these two proteins and genes between EC109 and EC109/CDDP cells by Western blot and RT-PCR were in accordance with the 2-DE results, confirming the veracity of the 2-DE gel image data and providing a rationale for future functional studies of these proteins.

An investigation of the reported functions of each individual identified protein was made. According to the available information in the Universal Protein Resource (UniProt) and published literatures, these proteins could be divided into several major groups: endoplasmic reticulum (ER) proteins in response to stress, metabolic enzymes, proteins involved in DNA replication and repair, nucleotide binding proteins, calcium binding proteins, cytoskeletal proteins, etc (Table II).

Four proteins, TXNDC4 (SSP 2338), protein disulfide-isomerase A3 precursor (PDIA3, SSP 3415), DnaJ homolog subfamily C member 9 (DNAJC9, SSP 4118) and DnaJ homolog subfamily B member 11 precursor (DNAJB11, SSP 5204), located in the ER and involved in the ER stress response, were up-regulated in EC109/CDDP cells. Cisplatin was reported to induce ER stress (19), therefore, we assume that EC109/CDDP cells were under ER stress induced by cisplatin. TXNDC4 and PDIA3 are members of the thioredoxin protein family, which assists in oxidative protein folding so as to relieve the overloading of mis- or unfolded proteins (20,21). TXNDC4 and PDIA3 function in controlling the ER and cytosolic Ca^{2+} homeostasis (22,23), thus keeping the cells away from apoptosis brought about by the cellular Ca^{2+} overload or perturbation of intracellular Ca^{2+} compartmentalization (24). Up-regulation of TXNDC4 and PDIA3 in EC109/CDDP might confer the cell resistance to apoptosis induced by cytotoxic drugs through their modulation of both the ER stress and Ca^{2+} homeostasis. DNAJC9 and DNAJB11 are members of DNAJ/HSP40s family, which plays important roles in regulating cell proliferation, survival and apoptosis by serving as chaperones for HSP70s (25). The induction of HSP70 members under stress conditions is a major UPR protective response and has been best characterized for its role in contributing to resistance to a wide variety of chemotherapeutic agents (including taxol, docetaxol, cisplatin, etoposide and celetoxib) in multiple tumor types (26,27). DNAJB11 and DNAJC9 might play an indirect role in drug resistance through their regulation of HSP70s.

According to previous studies, ER stress synergizes with cisplatin to more efficiently kill tumor cells, although it induces protection against topoisomerase-II-targeted chemotherapeutic agents (28). This is partly contrary to the fact that EC109/CDDP cells showed resistance to both cisplatin and etoposide at the same time. Thus, we are not sure whether the ER stress could account for EC109/CDDP cells' multi-drug resistant phenotype or just some of the drugs tested in our previous work (14). A more comprehensive understanding of which drugs the ER stress alters sensitivity to is essential, and EC109/CDDP would serve as an important cell model in research on the relationship between ER stress and cancer drug resistance.

A large group of metabolic enzymes were found to express differentially between EC109/CDDP and EC109 cells. SSP 6305 up-regulated in EC109/CDDP 2-DE gels was identified as protein CTH1 and/or CTH2 by MS analysis. CTH is an enzyme that catalyzes the conversion of cystathionine into cysteine, which was further metabolized to yield glutathione (GSH) and metallothionein (MT). Previous studies have shown that repeated exposure to cisplatin induced MT and GSH *in vivo* and *in vitro* and produced resistance to cisplatin by metabolizing it to an inactive form (29). Furthermore, inhibiting CTH enzymatic activity could restore the antitumor activity of cisplatin (30). Alternative splicing of this gene results in two transcript variants encoding different protein isoforms CTH1 and CTH2. Levonen *et al* (31) found that CTH1 was the predominant variant in human cells, and only cells over-expressing CTH1 rather than CTH2 had increased activity. In our work, Western blot showed it was the up-regulation of CTH1, but not CTH2 that plays a part in EC109/CDDP cell drug-resistance.

Besides CTH, other enzymes identified in this experiment include the up-regulation of START domain-containing protein 3 (SSP3110), abhydrolase domain-containing protein 14B (SSP4005) and dihydroxyacetone kinase (SSP8517), and down-regulation of acetyl-CoA acetyltransferase and fatty acid-binding protein (SSP6209), phosphoserine phosphatase (SSP3117), isoform 1 of low molecular weight phosphotyrosine protein phosphatase (SSP5020) and adenylate kinase isoenzyme 2 (SSP8115) in EC109/CDDP cells. The differential expression of these enzymes between EC109 and EC109/CDDP cells might reflect the change of EC109/CDDP in metabolism to survive chronic environmental stresses, such as the drug attack.

Moreover, several proteins involved in DNA replication and repair (DNA polymerase subunit δ-2, single-stranded DNA-binding protein mitochondrial precursor and uniquitin-conjugating enzyme E2N), protein biosyntheses and degradation (ribosomal protein SA, eukaryotic translation initiation factor 2 subunit 1, eukaryotic translation initiation factor 1b, isoform long of eukaryotic translation initiation factor 4H, 26S proteasome non-ATPase regulatory subunit 9 and NEDD8-conjugating enzyme Ubc12), nucleotide binding [poly(rC)-binding protein, far upstream element-binding protein 1, α-enolase, splicing factor arginine/ serine-rich 1 and cellular nucleic acid binding protein], calcium binding (RCN1, annexin A1, protein S100-A4 and S100-A11), cytoskeletal proteins (tubulin ß chain, tropomyosin α-1 chain, profilin-1, vimentin and keratin 1) and a transmembrane protein (voltage dependent anion selective channel 2) have been identified to express differentially between EC109/CDDP and EC109 cells. Some of them have been identified by previous 2-DE comparative proteomic analyses in various drug-resistant cancer cell lines (13). However, their participation in cancer drug resistance has not been discussed or studied extensively. Thus, we cannot make any assumption or conclusion regarding their possible roles in EC109 and EC109/CDDP cells.

Although we have identified scores of proteins with potential involvement in EC109/CDDP drug resistance, it has

to be admitted that the comparative proteomic approach based on 2-DE has some inherent technical limitations, for example, the deficiency in detection of proteins with lower abundance, the separation and identification of hydrophobic membrane proteins and proteins with extreme mass or pI. Furthermore, most of the proteins identified in the proteomic analyses have not been validated for their expression changes using another approach such as Western blot and for their role in drug resistance using functional assays. In future studies, validation and function analysis of the results originated from proteomic researches are not only desirable, but essential.

In conclusion, we identified candidate proteins involved in multiple biological functions that were differentially expressed in the multi-drug resistant cell line EC109/CDDP compared to parental cell line EC109 by comparative proteomic approach based on 2-DE. Some of the identified proteins in our work have not been reported to be related to cancer drug resistance before, such as TXNDC4, and may motivate us to look into ESCC drug resistance in a fresh perspective and generate novel hypotheses in addition to the well-known mechanisms. Further functional analysis *in vitro* and validation in clinical ESCC samples of these putative proteins to make certain their roles and mechanism in ESCC chemotherapeutic resistance are necessary and underway.

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