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Genomic and Proteomic Profiling of the Cadmium Cytotoxic Response in Human Lung Epithelial Cells

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Abstract

Microarray and proteomic expression patterns in response to cadmium exposure were analyzed in human lung epithelial cells. Among 35,000 genes analyzed by cDNA microarray, 228 genes were up-regulated and 99 genes were down-regulated, based on a fold change cut-off value of ≥2. Combining two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-ToF-MS), 25 of 629 protein spots showed fold changes in expression ≥2 (17 up-regulated, 8 down-regulated). After comparing the cDNA microarray and proteomic analyses, only transglutaminase 2, translation elongation factor 1 alpha 1, and glyceraldehyde-3-phosphate dehydrogenase showed overlapping signals in the cDNA microarray and proteomic analyses, whereas the remaining differentially expressed proteins showed large discrepancies with respect to mRNA expression.

Keywords: Genomics, Proteomics, Cadmium, Human lung epithelial cells

Cadmium (Cd^{2+}) is a ubiquitous industrial and environmental pollutant that bioaccumulates in the upper levels of the food chain, including humans[1-3]. Considering its current rate of release into the environment, cadmium content in the human body is likely to increase in the future[4]. Cadmium has been shown to be an effective inducer of apoptosis, and also affects cell proliferation, differentiation, gene expression, and signal transduction. Moreover, cadmium appears to promote the generation of reactive oxygen species (ROS), inhibit DNA repair and DNA methylation, and disrupt E-cadherin-mediated cell-cell adhesion[5,6]. The inhibition of DNA repair represents a mechanism by which cadmium enhances genotoxicity and promotes tumor initiation. The major mechanisms of cadmium-induced gene expression include the modulation of signal transduction pathways via enhanced protein phosphorylation and the activation of transcription and translation factors[7]. Cadmium has also proven to be toxic in many organs, including the lung, kidney, liver, testis, brain, bone, and blood. Cadmium is a known lung carcinogen and has been implicated in the development of other pulmonary diseases, including emphysema and interstitial fibrosis[8]. Cadmium is classified as a category I carcinogen by the International Agency for Research on Cancer and the US National Toxicology Program.

The identification of genes that are differentially expressed in response to toxic cadmium exposure would provide a better understanding of its mechanism of action and facilitate the search for sensitive and specific biomarkers of cadmium exposure and susceptibility. However, mRNA expression does not necessarily predict the level of corresponding protein expression and does not account for post-translational modification. Thus, although gene microarrays offer insight into the expression of numerous genes within a cell type, proteomic analysis is required to determine global protein expression in the cell. In this study, genomic and proteomic expression profiles were compared to identify genes and proteins altered by cadmium exposure[9]. Our results may assist in the selection of biomarkers for cadmium exposure at the gene and protein levels.

Cadmium Cytotoxicity in Human Lung Epithelial Cells

The MTT assay was used to determine the relative cell viability of human lung epithelial cells (NCI-H292) following exposure to a range of concentrations of cadmium chloride for different exposure times. Cadmium chloride reduced cell viability in a concentration-dependent manner. As shown in Figure 1, exposure to 40 μM cadmium chloride for 12 h resulted in an approximate 25% decrease in cell viability. This concentration and exposure time were used in subsequent geno-

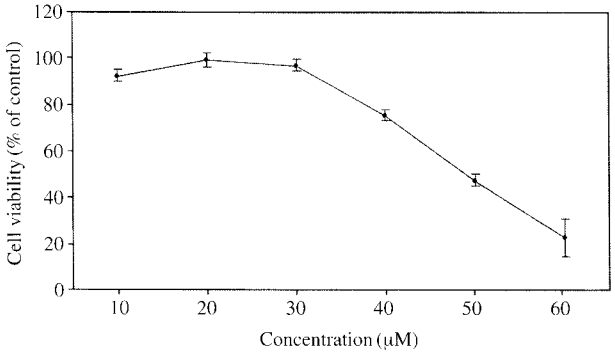

Figure 1. Effect of $CdCl_2$ on the viability of human lung epithelial cells (NCI-H292). The cells (5×10^4 cells/well) were treated with various concentrations of cadmium chloride for 12 h. The cell viability was determined by MTT assay.

mic and proteomic analyses.

cDNA Microarray Analysis

NCI-H292 cells were treated with 40 μM cadmium chloride for 12 h, and total RNA was subjected to microarray analysis. Changes in gene expression were analyzed by comparing the control group to the treated group using a two-fold balanced differential expression as the cut-off value with $P < 0.01$.

In this analysis, 228 genes were up-regulated and 99 genes were down-regulated after cadmium exposure. To analyze the underlying molecular mechanisms activated upon exposure to cadmium chloride, differentially expressed genes were classified according to their functions in terms of gene ontology and the KEGG pathway (Table 1). The majority of up-regulated genes were involved in cell proliferation, intracellular signaling, cell differentiation, cell cycle, transcription, signal transduction, development, transport, cell-cell adhesion, behavior, lipid metabolism, apoptosis, protein folding, cell adhesion, and the inflammatory and immune responses. The majority of down-regulated genes were involved in cell proliferation, intracellular signaling, cell differentiation, cell cycle, transcription, signal transduction, development, and transport.

Proteomic Analysis of Differential Protein Expression

To examine differential protein expression in human lung cells after cadmium chloride treatment, proteomic analysis was performed using high-resolution two-dimensional electrophoresis (2-DE). Figure 2 shows 2-DE images of human lung cell proteins following exposure to 40 μM cadmium chloride for 12 h. More than 629 protein spots with isoelectric points (pIs) between 3 and 10 and with relative molecular masses between 6.5 and 205 kDa were detected on the 2-DE gels.

After comparing 2-DE protein patterns on duplicate gels, we identified 25 protein spots that showed at least 2-fold differences in expression compared to basal levels (Table 2). Among these, 17 spots were up-regulated after 12 h of 40 μM cadmium exposure (Figure 3) and 8 spots were down-regulated (Figure 4). The up-regulated proteins included transglutaminase 2 (TG2) isoform a, prolyl 4-hydroxylase, keratin 8, translation elongation factor 1 alpha 1, protein phosphatase 1, tubulin, histone demethylase JARID1A, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ATP-dependent DNA helicase II, coated vesicle membrane protein, heterogeneous nuclear ribonucleoprotein, peroxiredoxin 1, porin 31HM, similar to *Homo sapiens* mRNA for KIAA0120 gene, and Nm23 human nucleoside diphosphate kinase B complexed with GDP. The down-regulated proteins included Tu translation elongation factor, chaperonin containing TCP1, HSPC140, acetyl-coenzyme A acetyltransferase 1, enolase 1, poly(rC) binding protein 1, human muscle L-lactate dehydrogenase M chain, and ATP synthase.

Comparative Analysis of Genomic and Proteomic Profiling

Comparison of the cDNA microarray and proteomic analyses showed large discrepancies between mRNA and protein levels (Table 3). Of the 25 up- or down-regulated proteins, only TG-2, translation elongation factor 1 alpha 1, and GAPDH showed appropriate corresponding changes in genes expression. Overall, a very low overlap was observed between the cDNA microarray and proteomic analysis data.

Discussion

Differentially expressed genes and proteins in human lung epithelial cells after cadmium treatment were investigated through genomic and proteomic approaches. This combined approach provides greater insight into the mechanisms underlying cadmium-induced toxicity.

Genomic approaches have improved the prediction of chemical carcinogenicity through the use of gene ontology analysis, which identifies the biological, cellular, and molecular functions of genes and thus provides insight into potential mechanisms leading to carcinogenesis. Proteomics is a powerful tool for the identification of differentially expressed proteins, which may then serve as biomarkers for cadmium toxicity in humans. Therefore, examining the global effects of cadmium cytotoxicity on gene and protein expression simultaneously helps to elucidate patterns of biological response, identify underlying toxic mechanisms, and discover candidate biomarkers.

Table 1. Identification of up- and down-regulated genes in $CdCl_2$-treated human lung epithelial cells.

Function	Genes
Up-regulation	
Cell Proliferation	MET (met proto-oncogene), VIP (vasoactive intestinal peptide), IL11, PBEF1 (Pre-B-cell colony enhancing factor 1)
Intracellular signaling cascade	DEDD2 (death effector domain containing 2), PLA2G4C (phospholipase A2, group IVC)
Cell differentiation	IL11, DHRS9, HOOK1 (Hook homolog 1)
Cell cycle	NEK1 (NIMA (never in mitosis gene a)-related kinase 1), RGS2
Transcription	MT1G, X, F, K, L (Metallothionein), MBD2, ZNF10, 165, 347, 483, 555 (zinc finger protein), ATF3, DEDD2 (death effector domain containing 2), POLR3E, FOXA3 (forkhead box A3), MAFF (v-maf musculoaponeurotic fibrosarcoma oncogene homolog F), TSC22D1, CREM, MED12
Signal transduction	CCRL2, EEF1A1, HRH1, TNFRSF8 (tumor necrosis factor receptor superfamily, member 8), CREM, MET, RASD1, GNA13, PBEF1
Development	FOSB (FBJ murine osteosarcoma viral oncogene homolog B), ARC, ARHGDIB, HOOK1, MET, GREM1 (gremlin 1)
Transport	SLC30A1, HBA1, SYT11, 13 (synaptotagmin XIII), ABCC1, 11 (ATP-binding cassette), RTN3 (reticulon 3)
Cell-Cell Adhesion	PKD1L1 (polycystic kidney disease 1 like 1), CD44, CLDN2 (claudin 2)
Behavior	FOSB
Lipid Metabolism	PLTP (phospholipid transfer protein), GBA
Apoptosis	PPP1R15A, SGPP1, BAG3 (BCL2-associated athanogene 3), RTN3 (reticulon 3), GAPDH
Protein folding	HSPA1A (heat shock 70 kDa protein 1A), TTC9B, HSPA1L, DNAJB1, HSPA8, BAG3, C1GALT1C1 (C1GALT1-specific chaperone 1), HSPB8, HSPA4
Cell adhesion	PCDH12 (protocadherin 12), SLAMF7 (SLAM family member 7), LAMC3, ITGA7, LY9, CD44, TGM2
Inflammatory response	MMP25, HRH1, PLA2G4C, F11R
Immune response	GEM (GTP binding protein overexpressed in skeletal muscle), ARHGDIB (Rho GDP dissociation inhibitor (GDI) beta), POU2F2
Down-regulation	
Cell proliferation	BIN1 (bridging integrator 1), CCDC88A (KIAA1212)
Intracellular signaling cascade	RACGAP1 (Rac GTPase activating protein 1), CDC42BPA (CDC42 binding protein kinase alpha), TENC1 (Tensin like C1 domain containing phosphatase), RASSF1
Cell differentiation	DAZAP1, BIN1 (bridging integrator 1)
Cell cycle	RACGAP1, NIPBL (Nipped-B homolog), RASSF1 (Ras association (RalGDS/AF-6) domain family 1)
Transcription	ZNF433 (zinc finger protein 433), IRF3, TEAD4 (TEA domain family member 4), POLR2C
Signal transduction	RACGAP1, IRS4 (insulin receptor substrate 4), CHRNA5 (cholinergic receptor, nicotinic alpha polypeptide 5), IGFBP5 (insulin-like growth factor binding protein 5)
Development	HOXD4 (homeobox D4)
Transport	MYO5A (myosin VA), SLC45A1 (solute carrier family 45, member 1)

In our comparative analysis, only TG-2, translation elongation factor 1 alpha 1, and GAPDH showed perfect overlap in terms of cadmium-induced mRNA and protein expression. The remaining differentially expressed proteins did not show good overlap with mRNA expression, highlighting the large discrepancies between mRNA and protein expression levels. Recently, large discrepancies between mRNA and protein levels after comparison of cDNA microarray and proteomic data have been reported[10]. In human carcinoma cells transfected with the HPV-16 E6 gene, large discrepancies between mRNA and protein expression were reported; similar to the present study, only three of numerous differentially expressed genes showed corresponding protein expression[11]. In human diploid fibroblasts exposed to H_2O_2, only a small fraction

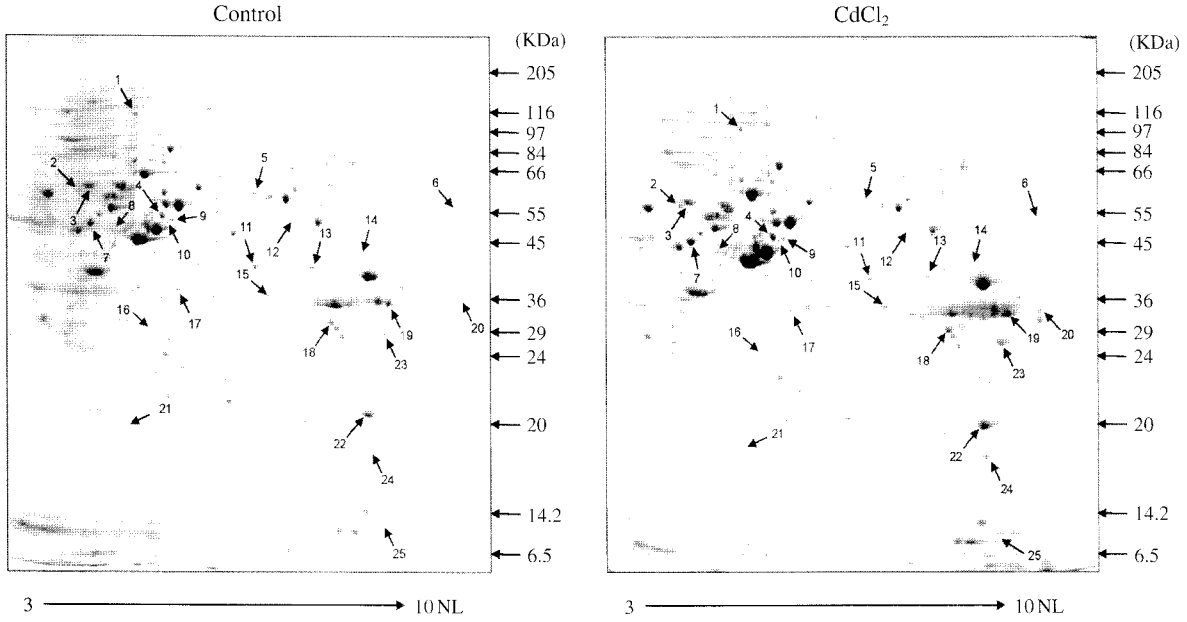

Figure 2. 2-DE image showing the protein spots from 40 μM $CdCl_2$-treated human lung epithelial cells.

of genes and proteins showed overlap in terms of microarray and proteomic analyses[12]. Moreover, a discrepancy between catalase gene transcription and enzyme activity has also been reported in *Haematococcus pluvialis* under stressful conditions[13].

There are several possible explanations for the low degree of overlap between data gained through microarray *versus* proteomic analysis. First, mRNA expression analysis provides little information regarding activation state, post-translational modification, alternative splicing, or the localization, selective degradation, or secretion of the corresponding proteins. In addition, differences in assay methodologies and detection sensitivity may also contribute to discrepancies in mRNA *versus* protein expression.

Interestingly, the proteomic data presented here do not support a role for metallothioneins, which are thought to provide protection against metal toxicity and oxidative stress, in the response to cadmium exposure. Microarray analysis revealed that metallothionein genes were strongly up-regulated (approximately 64-fold) after cadmium exposure (Table 1); in contrast, changes in protein expression were not apparent upon proteomic analysis. However, most metallothioneins are secreted proteins, which may explain why they were undetectable in a proteomic analysis using cell lysates. Thus, further proteomic analysis using medium containing secretory proteins seems to be needed to detect metallothionein.

TG2, translation elongation factor 1 alpha 1, and GAPDH showed perfect overlap in cDNA microarray and proteomic analyses in response to cadmium exposure (Table 3). TG2 is a ubiquitously expressed enzyme capable of catalyzing the formation of protein cross-links. These TG2-dependent cross-links are important to maintain extracellular matrix integrity, and it has been proposed that TG2 activity poses a barrier to tumor spread[14]. Epidermal growth factor-induced TG2 expression enhanced the oncogenic potential and chemoresistance in human breast cancer cells[15]. It has been shown that the expression of a dominant-negative form of TG2 reverses EGF-mediated chemoresistance in breast cancer cells, although the role of TG2 in the development of these tumor-related phenotypes remains to be elucidated[15].

The expression of translation-regulating genes, such as translation initiation factor 3 (TIF3) and translation elongation factor 1-delta (TEF-1δ), has been reported in several cadmium-treated cell types[16]. The oncogenic potential of both TIF3 and TEF-1δ were demonstrated in cadmium-transformed BALB/c-3T3 cells using antisense mRNAs against these factors[17]. Thus, cadmium-induced transformation and tumorigenesis may be, at least in part, due to the up-regulation of translation factors. Therefore, the overexpression of translation elongation factor 1 alpha 1 at the gene and protein levels may result in cadmium-induced transformation in NCI-H292 cells.

The housekeeping enzyme GAPDH has been reported to have both glycolytic and non-glycolytic functions. The proposed non-glycolytic functions of GAPDH include roles in endocytosis, microtubule bun-

Table 2. Identification of up- and down-regulated protein in $CdCl_2$-treated human lung epithelial cells.

Function	Spot No.	Protein Identified	Accession No.	Cov. %	Matching peptide No.	M.W/pI	Change
Up-regulated proteins							
Metabolism	2	prolyl 4-hydroxylase, beta subunit precursor	NP_000909	35	16	57.50/4.8	↗
	15	glyceraldehyde-3-phosphate dehydrogenase	CAA25833	32	8	36.21/8.4	↗
	16	ATP-dependent DNA helicase II	NP_066964	29	12	83.26/5.5	↗
	18	glyceraldehyde-3-phosphate dehydrogenase	CAA25833	45	12	36.21/8.4	↗
	20	glyceraldehyde-3-phosphate dehydrogenase	NP_002037	44	16	36.21/8.7	↗
	24	similar to Homo sapiens mRNA for KIAA0120 gene	AAG01993	55	12	24.61/8.6	↗
	25	Chain A, X-Ray Structure Of Nm23 Human Nucleoside Diphosphate Kinase B Complexed With Gdp At 2 Angstroms Resolution	1NUE_A	63	9	17.26/8.8	↗
Signal transduction	1	transglutaminase 2 isoform a	NP_004604	31	20	78.45/5.1	↗
	4	Keratin 8	AAH73760	45	29	53.79/5.5	↗
	7	protein phosphatase 1, regulatory subunit 7	NP_002703	22	4	41.66/4.8	↗
	17	coated vesicle membrane protein	NP_006806	24	7	22.86/5.1	↗
Cell structure	9	tubulin, beta	NP_821133	34	16	50.11/4.8	↗
Transcriptional regulation	11	Histone demethylase JARID1A	P29375	12	17	198.77/6.4	↗
Translation	6	translation elongation factor 1 alpha 1-like 14	AAK93966	26	7	43.29/9.1	↗
	19	heterogeneous nuclear ribonucleoprotein A2/B1 isoform A2	NP_002128	52	18	36.05/8.7	↗
Redox controlling	22	peroxiredoxin 1	NP_002565	47	15	22.32/8.7	↗
Enzyme regulator	23	Porin 31HM	AAB20246	62	16	30.74/8.8	↗
Down-regulated proteins							
Metabolism	10	acetyl-Coenzyme A acetyltransferase 1 precursor	NP_000010	30	12	45.47/9.2	↘
	12	enolase 1	NP_001419	29	12	47.49/7.0	↘
	21	ATPsynthase, H^+ transporting, mitochondrial F0 complex, subunit d isoform b	NP_001003785	62	8	15.81/6.6	↘
Signal transduction	3	Tu translation elongation factor, mitochondrial precursor	NP_003312	37	14	50.20/7.3	↘
	14	Chain A, Human Muscle L-Lactate Dehydrogenase M Chain, Ternary Complex With Nadh And Oxamate	1I10_A	42	18	36.82/8.7	↘
Chaperones	5	chaperonin containing TCP1, subunit 2	NP_006422	39	17	57.81/6.0	↘
Protease	8	HSPC140	AAF29104	38	15	38.86/5.0	↘
Protein folding	13	poly(rC) binding protein 1	NP_006187	37	10	38.02/6.7	↘

dling, nuclear RNA export, DNA replication, DNA repair, viral pathogenesis, and apoptosis[18]. In RGC-5 cells, GAPDH might play a notable role in apoptosis through relocalization within the subcellular compartments following oxidative stress, such as high pressure[19] and the addition of an NO donor[20]. Similarly, increased GAPDH expression appears to be associated with cell death due to cadmium toxicity in the present study.

In this investigation, we used genomic and proteomic approaches to identify factors that are differentially expressed in human lung epithelial cells in response to cadmium exposure. Although gene expression data at mRNA level have enhanced the understanding of the response mechanism of cells to Cd, many questions regarding the functional translated portions of the genomes under Cd toxicity remain unanswered. The functions of proteins depend considerably on post-translational modification and protein-protein interaction, which cannot be deduced from nucleic acid data. Genomic and proteomic profiling might offer a new platform for understanding complex biological networks under Cd toxicity, and serve as a key tool for dissecting the functional molecular mechanisms for the interactions between toxic metals and cells.

The present research might be helpful to understand

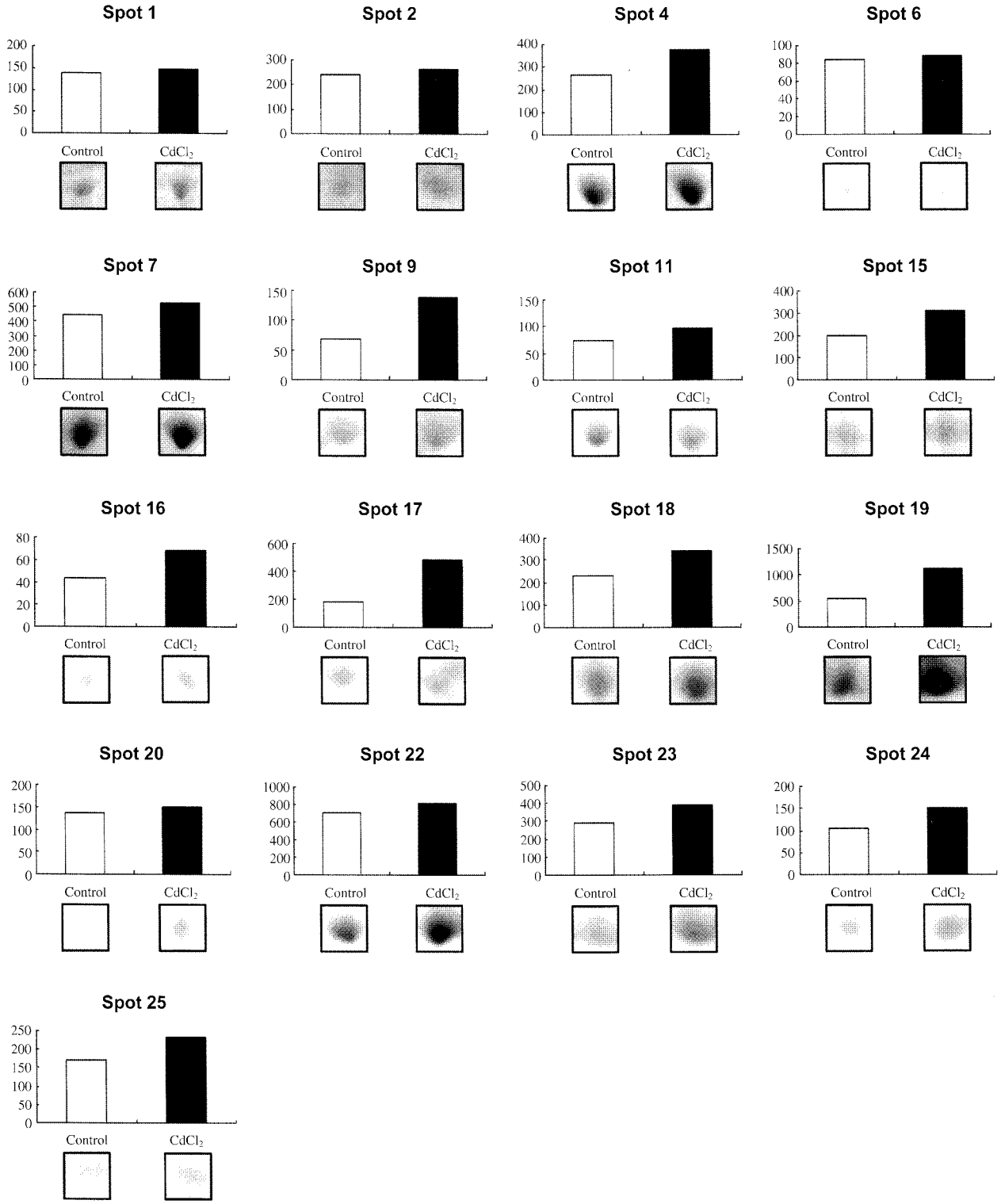

Figure 3. Up-regulated proteins from human lung epithelial cells by $CdCl_2$. Protein expression levels were determined by relative intensity using image analysis.

toxic Cd-induced dynamic changes in the genome and proteome and, ultimately, to unravel how their expressions are controlled within regulatory networks. Moreover, this combined approach provides deeper insight

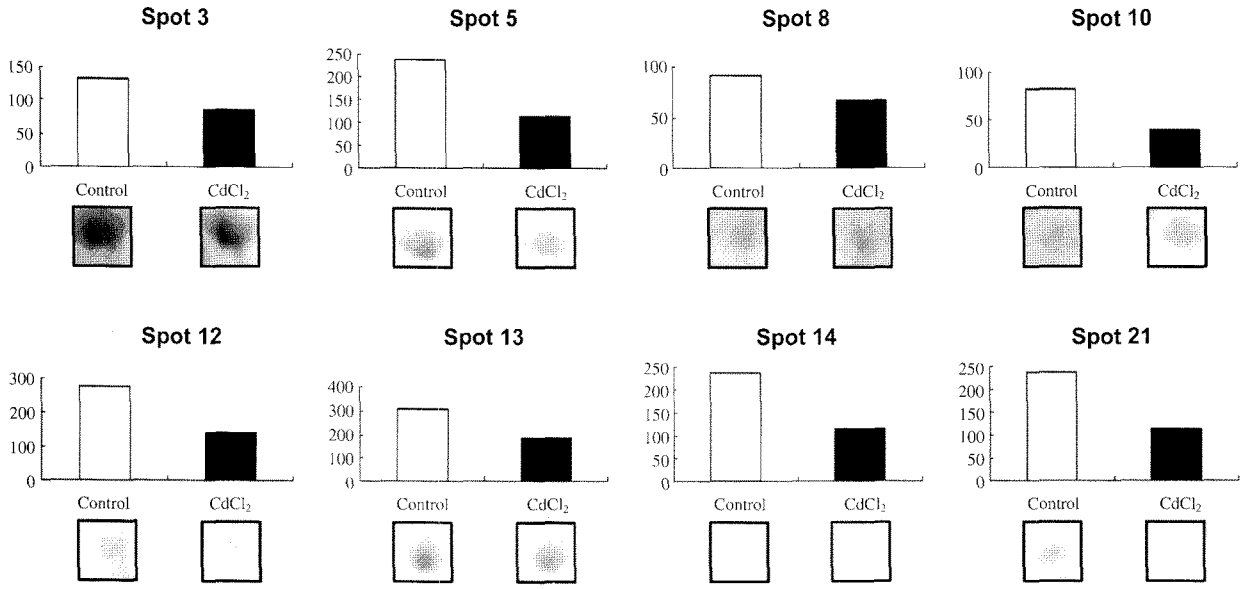

Figure 4. Down-regulated proteins from human lung epithelial cells by $CdCl_2$. Protein expression levels were determined by relative intensity using image analysis.

Table 3. Identically matched genes and proteins in human lung epithelial cells by cDNA microarray and proteomics.

Identified protein	Accession No.	Cov. %	Matching peptide	M.W/pI	Change	Identifued gene	GenBank Accession No.	Gene Symbol	Change
transglutaminase 2 isoform a	NP_004604	31	20	78.45/5.1	↗	transglutaminase 2 (C polypeptide, protein-glutamine-gamma-glutamyltransferase)	NM_198951	TGM2	↗
translation elongation factor 1 alpha 1-like 14	AAK93966	26	7	43.29/9.1	↗	Eukaryotic translation elongation factor 1 alpha 1	CR599412	EEF1A1	↗
						Eukaryotic translation elongation factor 1 alpha 1	BI160776	EEF1A1	↗
glyceraldehyde-3-phosphate dehydrogenase	CAA25833	32	8	36.21/8.4	↗	similar to Glyceraldehyde 3-phosphate dehydrogenase	XM_497382	GAPDH	↗
glyceraldehyde-3-phosphate dehydrogenase	CAA25833	45	12	36.21/8.4	↗	similar to Glyceraldehyde 3-phosphate dehydrogenase	BX378750	GAPDH	↗
glyceraldehyde-3-phosphate dehydrogenase	NP_002037	44	16	36.21/8.7	↗				

into cadmium cytotoxicity and may facilitate the discovery of biomarkers for cadmium exposure at the gene and protein levels.

Materials & Methods

Human Lung Epithelial Cells and Cadmium Chloride Treatment

Human lung epithelial cells (NCI-H292) were maintained in RPMI-1640 containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were cultured at 37°C in 150-mm dishes (density, 3.7 $\times 10^6$ cells per dish) under a humidified atmosphere of 5% CO_2 in 95% air. The cells were then treated with 40 μM cadmium chloride for 12 h.

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on RNA samples from NCI-H292 cells using Human Whole 35K microarray chips (Operon Biotechnologies, Inc., Germany)[21]. This was followed by coupling of Cy3

dye for the controls or Cy5 dye for the treated samples. Hybridization was performed in a hybridization oven at 62°C for 12 h. After washing (2 × SSC/0.1% SDS for 2 min at 58°C, 1 × SSC for 3 min at RT, and 0.2 × SSC for 2 min at RT), the slide was dried by centrifugation at 800 rpm for 3 min at RT. Hybridization images on the slides were scanned using a GenePix 4000B scanner (Axon Instruments, Union City, CA, USA). Scanned images were analyzed with GenePix 4.1 software (Axon Instruments) to obtain gene expression ratios.

Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. We used the robust scatter-plot smoother LOWESS function to perform intensity-dependent normalization for gene expression. Scatter plot analysis was performed using Microsoft Excel 2000 (Microsoft, WA, USA). Significance analysis of microarray (SAM) was performed to select genes with significant changes in gene expression[22]. The statistical significance of the differentially expressed genes was assessed by computing a q-value for each gene. To determine the q-value, a permutation procedure was used, and for each permutation, two-sample t-statistics were computed for each gene. Genes were differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 0.5 or less than −0.5 (*i.e.*, a 2.0-fold difference in expression level) and when the q-value was < 1.

Sample Preparation

Harvested NCI-H292 cells were extracted with lysis buffer containing 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, and 1 mM PMSF protease inhibitor. After centrifugation, the supernatant was collected and protein concentrations were determined using 10% trichloroacetic acid (TCA) in acetone. The protein pellet was washed in ice-cold acetone at least five times to remove contaminants.

2-Dimensional Gel Electrophoresis (2-DE)

2-DE was carried out as described previously[23-25]. For isoelectric focusing (IEF) in the first dimension, protein samples were dissolved in 500 μL of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholytes, 100 mM DTT, and 0.01% bromophenol blue). The sample solution was applied to an immobilized pH 3-10 nonlinear gradient drystrip using an IPGphor system (GE Healthcare Biosciences). IEF was performed using the following steps: rehydration for 12 h, 200 V/200 Vhr, 500 V/500 Vhr, 1,000 V/1,000 Vhr, 8,000 V/8,000 Vhr. After IEF, the individual IPG strips were equilibrated in 6 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 2% DTT, and 10% glycerol for 15 min and subsequently incubated in the same buffer for an additional 15 min after replacing DTT with 2.5% iodoacetamide. The gel was run at 20 mA for 15 min for the initial migration and then 60 mA for 5 h.

The gels were visualized using Coomassie brilliant blue G-250 (CBB-G250). The stained gels were scanned using a UMAX scanner (UMAX Technologies, Plano, TX, USA), and the data were analyzed using Image Master 2D Elite software (GE Healthcare Biosciences).

Protein Digestion and Mass Spectrometric Analysis

Differentially expressed protein spots were excised from the gel. Gel pieces were washed twice with 25 mM ammonium bicarbonate (pH 8.2) and 50% v/v acetonitrile (ACN), and then dehydrated by the addition of 100% CAN. Thirty nanograms of trypsin in 25 mM ammonium bicarbonate were added to each gel piece and the pieces were incubated at 30°C for 16 h. The peptide solution was automatically desalted, concentrated, and spotted onto Axima matrix-assisted laser desorption/ionization-time-of-flight-mass spectrometry (MALDI-ToF-MS) target plates (Kratos, Manchester, UK).

Peptide mass fingerprints and post-source decay (PSD) tryptic peptides were generated by MALDI-ToF-MS using Ettan MALDI-ToF system (GE Healthcare Biosciences). Tryptic peptides derived from protein spots were analyzed and amino acid sequences were deduced using a *de novo* peptide sequencing program, PepSeq (Gibbsland). To identify the proteins, sequences were searched against the NCBInr and EST databases using the PROFOUND search program (http://www.rockefeller.edu/labheads/chait/novel_tandem.php) and BLAST.

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