

# Proteomics Investigation Reveals Apoptosis-Associated Proteins in Aryl Hydrocarbon Receptor-Deficient Human Lung Cells Treated with 2,3,7,8-Tetrachlorobenzo-p-dioxin

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## Abstract

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), the most potent dioxin, binds to aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor, which subsequently alters gene expression. Epidemiological studies suggested that exposure to TCDD highly enhanced the risk of pulmonary diseases. In this study, we adopted a comparative proteomic approach using two-dimensional gel electrophoresis (2DE) to identify the differential expression of proteins between TCDD-treated control and AhR-deficient lung adenocarcinoma cells. Fifteen proteins from seventeen differential protein spots were identified that four of which were down-regulated and eleven were up-regulated. Western blotting analysis confirmed differential expression for five identified proteins, including GRB10, YWHAG, HSP27, LGALS1 and ACTB. Further protein-protein interaction network analysis of the identified proteins illustrated that twelve proteins were linked to a network containing caspase-3, p53, MDM2 and Akt/PKB, which is known to be associated with apoptosis pathway. Among the identified proteins, GRB10 may play a critical role to regulate the development of apoptosis in human lung cells by TCDD exposure. According to our study, it is expected to lead to new insights into the apoptosis pathway in lung adenocarcinoma by TCDD exposure.

**Keywords:** 2,3,7,8-tetrachlorodibenzo-p-dioxin; Aryl hydrocarbon receptor; Proteomics; Apoptosis; GRB10; 2DE

## Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), a prototype of Polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs), is one of most dangerous pollutants in the environment due to its resistance to degradation in animal and human fats [1-3]. By industrialization, TCDD and other dioxin-like congeners as a by-product are unintentionally produced during the combustion of fossil fuels and woodland during the incineration of industrial wastes. In 1997, TCDD is classified as a group 1 carcinogen by the International Agency for Research on Cancer (IARC) according to limited evidence of carcinogenicity in humans and sufficient evidence in animals [4]. In epidemiological studies, people with TCDD exposure increase the risk of various cancers, such as liver cancer, non-Hodgkin's lymphoma, soft-tissue sarcoma, rectal cancer and lung cancer [5-7]. Some carcinogenic researches report that experimental animals treated different doses of TCDD have been applied comprehensively to uncover the mechanisms of TCDD-induced tumor promotion [8]. In our previous study, one of the mechanisms is oxidative stress induced by TCDD to lead to DNA mispairings [9]. These mutations cause the perturbation of oncogenes or tumor suppressor genes to result in the uncontrolled signal transduction pathways, i.e., NF- $\kappa$ B, AP-1, *c-fos*, *c-jun*, or p53 [10-11]. Therefore the tumor progression represents from hyperplasia to carcinoma. This indicates that oxidative stress plays a role in the mechanisms of carcinogenesis by TCDD. In the past decade, we continually monitored the PCDD/F levels of dietary intake, formula/breast milk, eggs and serum in Taiwanese [12-14]. A "hot spot" has been discovered in Taiwan to may help the future epidemiologic studies when the relationships between PCDD/F levels and health effects [15]. Therefore, it is important to understand the pathogenic mechanism of TCDD or other congeners.

Up to date, the molecular mechanisms of TCDD-mediated responses have been slightly cleared. The characterization of TCDD is as a high affinity ligand to interact with aryl hydrocarbon receptor (AhR), which is a cytosolic transcription factor of the basic helix-loop-helix/Per-Arnt-Sim family. Upon TCDD binding, the AhR complex translocates into nucleus and acts a concert with AhR nuclear translocator (ARNT). The activated AhR/ARNT heterodimer binds to DNA at xenobiotic-responsive elements (XREs) located and induced upstream the target genes of many drug-metabolizing enzymes including cytochrome P450 enzyme 1A1 (CYP1A1), CYP1B1 and CYP1A2 [4, 16-17]. In previous study, we have established the interactive effects between CYP1A1 genotypes and PCDD/Fs exposure containing TCDD on liver function profile [18]. Furthermore, we also utilized the mouse model to establish the casual relationship of AhR expression, AhR activation (CYP1A2 expression up-regulation) and hepatic toxicity [19]. Consequently, AhR plays an important role for detoxification pathway depended on the high-affinity of environmental contaminants. Although the AhR in human is widely expressed in liver, kidney, thymus and lung, the

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research core of AhR mediated with TCDD focus on liver development, which is high expression of drug-metabolizing enzymes. Besides central role of detoxification, many studies demonstrated that AhR also participates in other signal transduction pathways to influence the biological processes of cell cycle regulation, oxidative stress response and apoptosis [20-22]. However, only few studies emphasized that the TCDD-mediated AhR influences the mechanisms associated with lung diseases [23-24]. In recent years, exposure to TCDD that accompanied over-expression of CYP1A1 and CYP1B1 has been implicated in the pathogenesis of lung adenocarcinoma [25]. In our previous studies, the expression level of AhR in the lung adenocarcinoma is up-regulated [26]. In further analysis, the AhR-deficient lung adenocarcinoma H1355 cell line by RNA interference technique is constructed to investigate the regulation of intracellular oxidative stress and cell growth [27]. However, the relationship of AhR and TCDD exposure in lung cancer is not clear until now. The objective in this study is to utilize the proteomic approach to research various molecular mechanisms and potential proteins involved in TCDD-mediated AhR signaling pathway of lung adenocarcinoma cells.

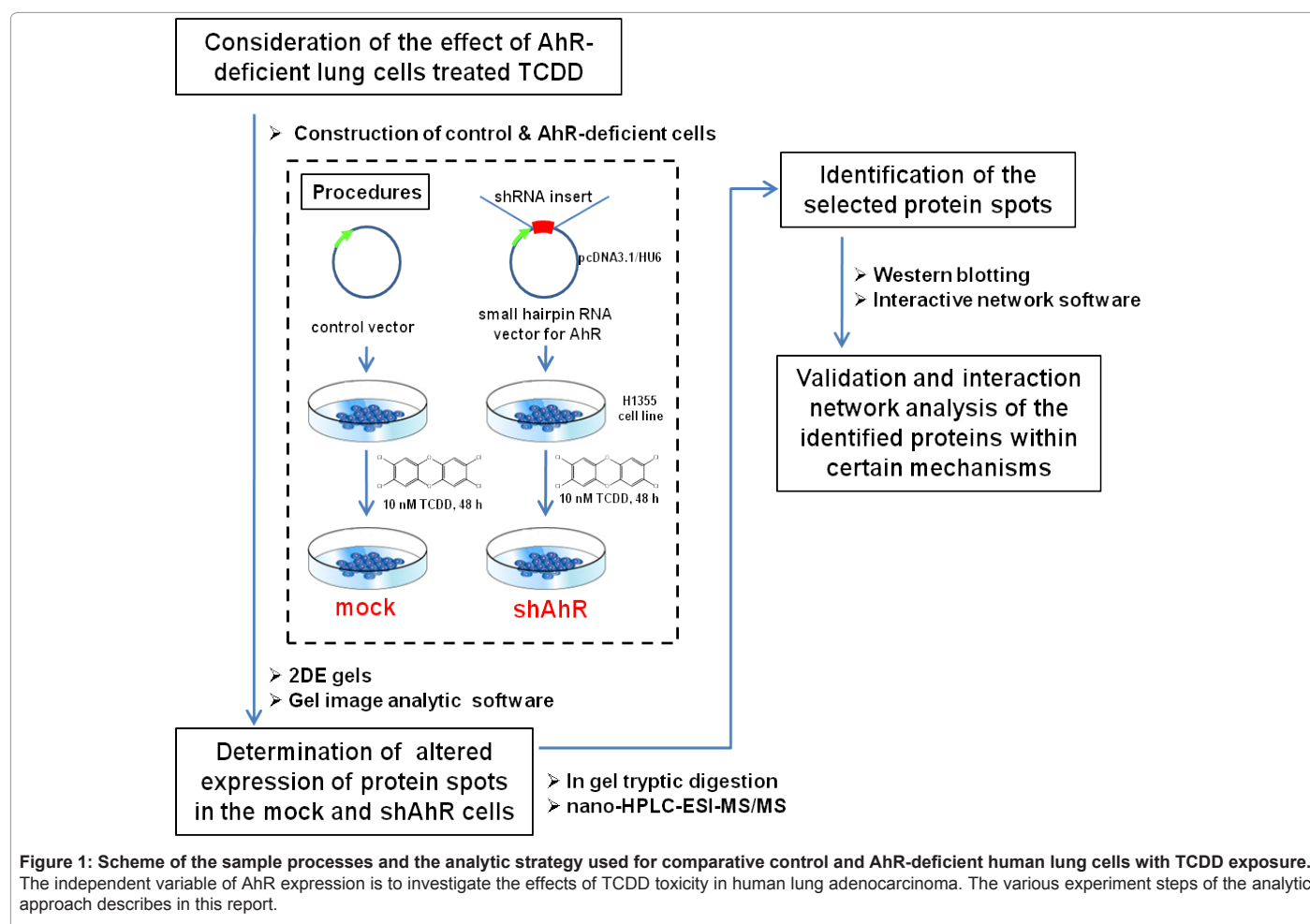
At present, the comparative proteomic approach is considered to be important for the system-based understanding of the molecular function of each identified protein between different treatments at the same conditional background [28]. The differently expression of

proteins are expected to provide insights into the various molecular mechanisms. Previously, the major proteomic studies are emphasized that the aspect of liver lesion is underlying TCDD-induced toxicity according to the modulation of drug-metabolizing enzymes [29-31]. Although the lung lesion and cancer are considered to associate with TCDD exposure, the systemic proteomic analysis has not been reported. In this study, we performed that one of a control vector (mock) was transfected into H1355 cells to compare with the other of a stably expressed short hairpin RNA vector for AhR (shAhR) using two-dimensional gel electrophoresis (2DE) coupled with mass spectrometry (MS). Figure 1 contains the experimental design developed for this study. The differential expression of identified proteins in mock and shAhR cells are considered to be involved in some biological processes and then the candidate proteins may be as the potential targets for therapy or the possible markers for diagnosis.

## Materials and Methods

### Cell culture and TCDD treatment

There were two human lung adenocarcinoma cell types to be constructed as previous described [27]. One was H1355 cell transfected with a control vector (mock) and the other was transfected a stably expressed short hairpin RNA for aryl hydrocarbon receptor vector (shAhR) and then all maintained in a RPMI-1640 medium (Gibco BRL, Gaithersburg, MD). The medium was supplemented with 5% heat-



**Figure 1: Scheme of the sample processes and the analytic strategy used for comparative control and AhR-deficient human lung cells with TCDD exposure.** The independent variable of AhR expression is to investigate the effects of TCDD toxicity in human lung adenocarcinoma. The various experiment steps of the analytic approach describes in this report.

inactivated fetal bovine serum at 37°C under 5% CO<sub>2</sub>. A total 5×10<sup>5</sup> cells of mock and shAhR, plated on respective 10 cm cell culture dishes, were treated with 10 nM TCDD when cultures were 70-80% confluent. After incubation for 48 h, these cell lines were collected and then lysed in NP-40 lysis buffer for 30 min at 4°C. The cell lysates were centrifuged at 12,000 g for 30 min. The resulting supernatants were collected and stored at -20°C.

### Protein preparation and 2DE

Cell extracts were subjected to 10% thichloroacetic acid in ice cold acetone at -20°C overnight and then precipitated proteins were collected by centrifugation (16,000 g, 4°C, 20 min). The protein pellet was washed with 100% ice cold acetone and allowed to air dry. Protein concentration was evaluated using the Bradford protein assay (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. The 100 µg protein amounts were dissolved in rehydration buffer for isoelectric focusing (IEF) analysis. Immobilized pH gradient (IPG) strips of p<sup>H</sup> 4-7 and length 18 cm (IPGphor, GE health, Uppsala, Sweden) were focused on a stepwise incremental voltage program: 30V for 12h, 500V for 1h, 1000 V for 1h and 8000 V for 5h, with a total power of 42 kWh. After IEF, the strips were subjected to a two-step equilibration in equilibration buffer I (6 M urea, 30% glycerol, 2% SDS and 50 mM Tris-HCl; p<sup>H</sup> 8.8) containing 1% w/v dithiothreitol (DTT) followed by equilibration buffer II containing 2.5% w/v iodoacetamide (IAA) for 15 min, respectively. Proteins were separated in gradient (8-16%) polyacrylamide gels using a PROTEAN II xi system (Bio-Rad, Hercules, CA) at 15°C. Following the gels into a fixation solution, the proteins in gels were detected by silver staining [32].

### Gel image analysis

The stained gels were scanned with resolution of 300 dpi using ImageScanner and LabScan software (GE health, Piscataway, NJ). The resulting gel images were analyzed with ImageMaster 2D Platinum software. The detection of protein spots, subtraction of background and spot matching were performed automatically and manually. After normalization for spot intensity in each 2DE-gel by computerized software, the spot intensity ratio of shAhR cells is divided by mock cells. The 95% confidence interval (CI) of spot intensity ratio was calculated from six replicates. The lower confidence limit was greater than 1 and the upper confidence limit was smaller than 1 to be determined as up-regulated and down-regulated, respectively. Each experimental ratio from six replicate 2DE analyses of mock and shAhR cells was performed with *t*-test. The *p* < 0.05 was considered statistically significant. The protein spots with significantly altered levels were excised for identification by mass spectrometry.

### Tryptic In-Gel digestion

Excised protein spots were destained with 1:1 solution of 30mM potassium ferricyanide and 100 mM sodium thiosulfate until colorless at room temperature for 15 min. The destained spots were washed with 1:1 solution of acetonitrile (ACN) and 25 mM ammonium bicarbonate. The spots were reduced with 10 mM DTT for 1 h at 56°C and alkylated with 55 mM IAA for 45 min in the dark at room temperature. Subsequent digestion was performed using 0.1 µg of TPCK-treated modified porcine trypsin (Promega, Madison, WI) overnight at 37°C. The supernatant containing tryptic peptides was combined with two more extracts of the pieces by 50% ACN/5% formic acid (FA) and

then dried using a centrifugal evaporation instrument (Vacufuge, Eppendorf, Hauppauge, NY).

### MS/MS analysis and database searching

The dried peptides suspended with 1% FA/5% ACN were analyzed by nano-HPLC-ESI-MS/MS. The peptides were separated by a nano-HPLC system (LC Packings, Amsterdam, Netherlands) connected to an ion trap mass spectrometer (LCQ DECA XP Plus, ThermoFinnigan, San Jose, CA) equipped with an electrospray ionization source. Peptide mixtures were loaded onto a 75 µm I.D., 150 mm length C18 microcapillary column packed with 3 µm particles with a pore of 100Å and separated using a segmented gradient in 40 min from 0% to 60% solvent B (80% ACN with 0.1% FA) in solvent A (5% ACN with 0.1% FA) at a flow rate of 200 nl/min. Peptides were electrosprayed into the LCQ with the application of a distal 1.3 kV spraying voltage. Each cycle of one full scan MS spectrum (*m/z* 450-2000) was followed by three tandem mass spectra with collision energy set at 35%.

All MS and MS/MS raw files were processed by Bioworks Browser 3.1 (ThermoFinnigan, San Jose, CA) and searched against the human protein sequence database (Swiss-Prot) via an in-house Mascot server (Matrix Science Ltd, London, UK). Carboxyamidomethylation on cysteine and oxidation on methionine residues were set as variable modification. Search criteria used were trypsin digestion and allowing up to 2 missed cleavages. The mass tolerances of parental and fragment ion were all set to 1 Da. MOWSE scores of greater than 38 were considered significant (*p* < 0.05).

### Bioinformatics analysis

To functionally annotate the identified proteins, the cellular components and biological processes were described in the Human Protein Reference Database (HPRD) [33]. For pathway analysis, the accession numbers of identified proteins were uploaded and analyzed for evidence of a protein-protein interaction network using an integrated knowledge and software suite, MetaCore (GeneGo, Inc., St. Joseph, MI). The existing canonical map was generated by an add-on tool, MapEditor, which integrated with MetaCore.

### Western blotting

Proteins extracted from the cell lysates of mock and shAhR cells with 10 nM TCDD were resolved by SDS-PAGE using 10% polyacrylamide. The proteins were mixed with an equal volume of 2× sample buffer according to the suggestion in the Bio-Rad instruction manual and the mixtures were boiled for 10 min. After electrophoresis, the separating gels were transferred onto PDVF membranes (Pall Corp., East Hills, NY) in a Bio-Rad Trans-Blot system (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. These were blocked with TBST buffer (20 mM Tris-HCl, 0.5 M NaCl, 0.1% Tween 20, p<sup>H</sup> 7.6) containing 5% skim milk for 1 h at room temperature. The membranes were incubated with primary antibodies against the proteins of cell lysates (GRB10, YWHAG, HSP27, LGALS1, ACTB and GAPDH) overnight at 4°C, washed three times with TBST buffer and incubated with secondary antibodies conjugated with horseradish peroxidase (HRP) for 1 h at room temperature. After the membranes had been washed three times with TBST buffer, the protein bands were developed with enhanced chemiluminescence (ECL) detection.

### Statistical analysis

The relationship of significantly altered protein levels between mock and shAhR cells were normalized to GAPDH levels. Differences



in mean values between the two groups were determined using *t*-test. The  $p < 0.05$  was considered statistically significant.

## Results

### Comparative protein quantitation of mock and shAhR cells treated with TCDD by 2DE

H1355 cells, a kind of lung adenocarcinoma cell line, were transfected with a control vector (mock) and a stably expressed short hairpin RNA for aryl hydrocarbon receptor vector (shAhR). The protein expression level of aryl hydrocarbon receptor (AhR) in shAhR cells was reduced to 30% of mock cells as previous described [27]. The protein

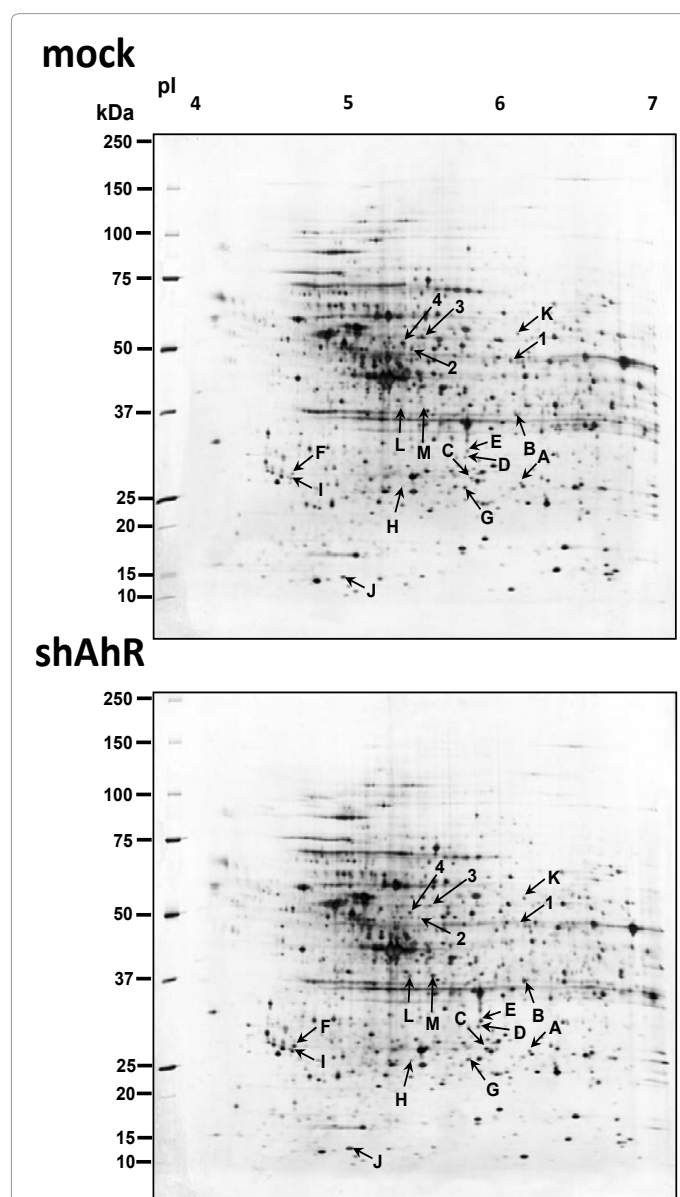
expression profiles in mock and shAhR cells with TCDD treatment were obtained by 2DE with linear IPG ranging from  $pH^1$  4-7 (Figure 2). The six replicates of samples from mock and shAhR cell lysates were performed, respectively. The relative intensities of the normalized spot volumes were analyzed and compared between mock and shAhR cells using ImageMaster 2D Platinum software. Approximately 500 spots were detected on each gel with molecular weight ranging from 10 to 150 kDa and  $p^1$  value between 4.5 and 7. After software process, spot intensity presented in gels was calculated. A ratio was determined that the spot intensity of the calculation of shAhR cells was divided by mock cells, the calculation of shAhR cells was divided by mock cells and then the confidence interval (CI) of ratio was calculated from the six replicated pairs. The spot variation between these two cell lines was determined when the lower confidence was greater than 1 as up-regulated or the upper confidence was smaller than 1 as down-regulated. Consequently, there were seventeen differential protein spots to be identified that thirteen protein spots were up-regulated and four was down-regulated. The protein spots differentially expressed in mock and shAhR cells were excised and then subjected to further analysis.

### Identification of variant protein spots in 2DE maps of mock and shAhR cells

After MS/MS analysis and database searching, a total of fifteen proteins in seventeen protein spots were identified that four proteins were as down-regulated and eleven proteins were as up-regulated in mock cells compared with shAhR cells (Table 1). The two spot numbers, G and H, were identified as heat shock protein beta-1 (HSP27) and the other two, L and M, were also identified as beta-actin (ACTB). The close-up images of seventeen identified protein spots with differentially expression in mock and shAhR cells were presented in Figure 3. For further functionally annotation, the fifteen identified proteins were categorized to cellular components and biological processes according to the HPRD website (<http://www.hprd.org/query>) (Figure 4). The largest proportion of identified proteins was to be located in the cytoplasm. In the classification of biological processes, many of the identified proteins were functioned into metabolism and signal transduction.

### Validation and Interaction Network Analysis of Identified Proteins

Regarding the result of proteomic analysis, the five identified proteins revealed by Western blotting and the result concurred with comparative protein quantization in 2DE (Figure 5). There were one protein, GRB10, higher expression in mock cells and the other four proteins, YWHAG, HSP27, LGALS1 and ACTB, higher in shAhR cells. Band intensity was measured and the relative intensity ratio to corresponding GAPDH band was calculated. After statistical analysis of triplicate, the results of fold change showed a consistent trend in protein abundance in comparative 2DE gels. The description of protein-protein interaction was analyzed by analytic software, MetaCore, loading the Swiss-Prot accession numbers of fifteen identified proteins with altered expression level between mock and shAhR cells. The interactive map was illustrated by the software, MapEditor, integrated into MetaCore according to proteins' cellular localizations (Figure 6). The processed result showed that the twelve of fifteen identified proteins were involved in the generated network containing 29 proteins. The interactive network also includes the significant apoptosis-associated proteins such as caspase-3, p53, MDM2 and Akt/PKB. The map illustrated that the eight identified proteins of GFAP, HSP60, GRB10,

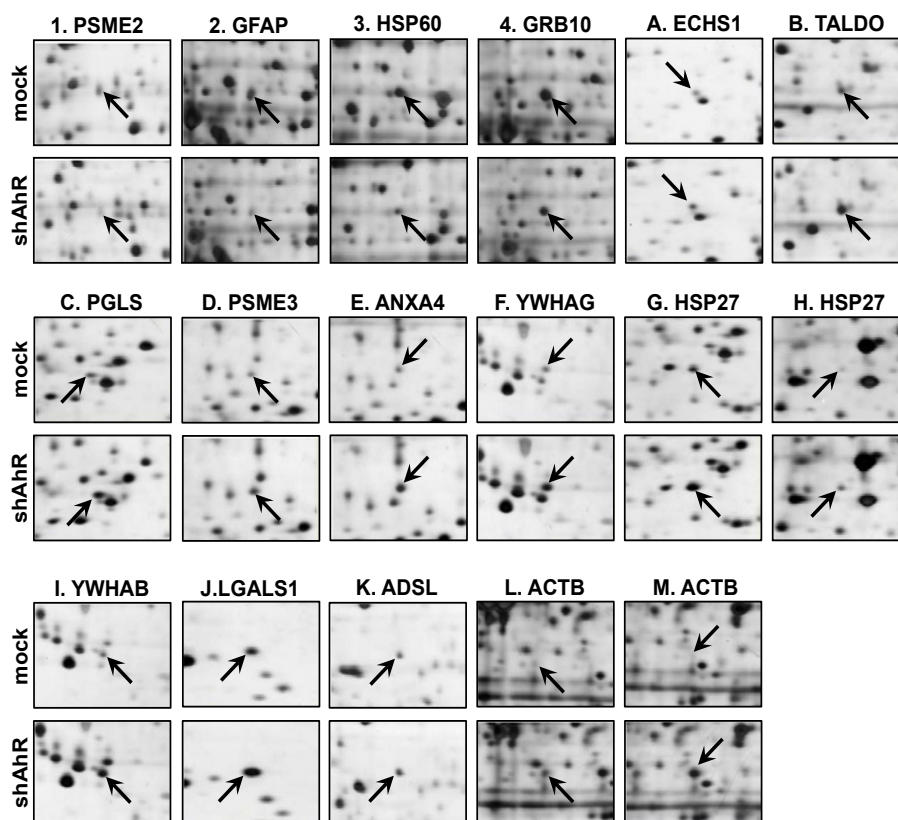


**Figure 2: Representative 2DE gel maps for mock and shAhR cells.** A total of 100  $\mu$ g of proteins were separated by 2DE using the first dimension of 18 cm pH 4-7 linear strips and the second dimension of 8-16% gradient SDS-PAGE. Separated proteins were visualized by silver staining. The spots were analyzed with ImageMaster software and identified by MS/MS sequencing. Down-regulated and up-regulated proteins are labeled as Arabic numerals and English alphabet, respectively.

Spot no. <sup>a</sup>	Accession no. <sup>b</sup>	Gene name	Protein name	Mr (E/T) <sup>c</sup> (kDa)	pI (E/T) <sup>d</sup>	Score	Matched peptides	Ratio average <sup>e</sup>	Regulation shAhR/mock <sup>f</sup>
1	Q9UL46	PSME2	Proteasome activator complex subunit 2	48.5/27.2	6.01/5.44	111	3	0.156	down
2	P14136	GFAP	Glial fibrillary acidic protein	51.5/49.9	5.38/5.42	76	3	0.218	down
3	P10809	HSP60	60 kDa heat shock protein, mitochondrial	53.2/58.0	5.44/5.24	129	7	0.407	down
4	Q13322	GRB10	Growth factor receptor-bound protein 10	52.2/67.2	5.30/8.06	48	4	0.518	down
A	P30084	ECHS1	Enoyl-CoA hydratase, mitochondrial	27.5/28.3	6.15/5.88	153	3	1.24	up
B	P37837	TALDO	Transaldolase	36.8/37.5	6.10/6.36	135	2	1.97	up
C	O95336	PGLS	6-phosphogluconolactonase	27.5/27.4	5.75/5.71	341	8	2.06	up
D	P61289	PSME3	Proteasome activator complex subunit 3	30.5/22.8	5.73/5.98	230	3	2.14	up
E	P09525	ANXA4	Annexin A4	31.5/35.8	5.74/5.85	50	3	2.19	up
F	P61981	YWHAG	14-3-3 protein gamma	28.2/28.3	4.60/4.80	125	8	2.44	up
G	P04792	HSP27	Heat shock protein beta-1	27.1/22.8	5.73/5.98	235	13	2.56	up
H	P04792	HSP27	Heat shock protein beta-1	26.1/22.8	5.31/5.98	171	3	6.29	up
I	P31946	YWHAB	14-3-3 protein beta/alpha	27.8/28.0	4.58/4.76	372	16	2.86	up
J	P09382	LGALS1	Galectin-1	14.5/14.6	4.95/5.34	172	8	3.23	up
K	P30566	ADSL	Adenylosuccinate lyase	56.2/54.8	6.10/6.73	39	5	7.43	up
L	P60709	ACTB	Actin, cytoplasmic 1	38.1/41.7	5.30/5.29	118	2	25.5	up
M	P60709	ACTB	Actin, cytoplasmic 1	37.1/41.7	5.48/5.29	142	3	47.2	up

<sup>a</sup> Spot number corresponds to the labels in Figure 2 and 3. <sup>b</sup> Accession number was from Swiss-Prot database. <sup>c</sup> Mr (E/T): experimental observation of apparent molecular weight in 2-DE/theoretical molecular weight calculated from protein sequence database. <sup>d</sup> pI (E/T): experimental observation of pI in 2-DE/theoretical pI calculated from protein sequence database. <sup>e</sup> The ratio average was calculated with six duplicates of shAhR cells divided by mock cells. <sup>f</sup> According to ratio average, smaller than 1 was as down-regulated and greater than 1 was as up-regulated.

**Table 1:** Identification of Differentially Expressed Proteins using Mass Spectrometry.

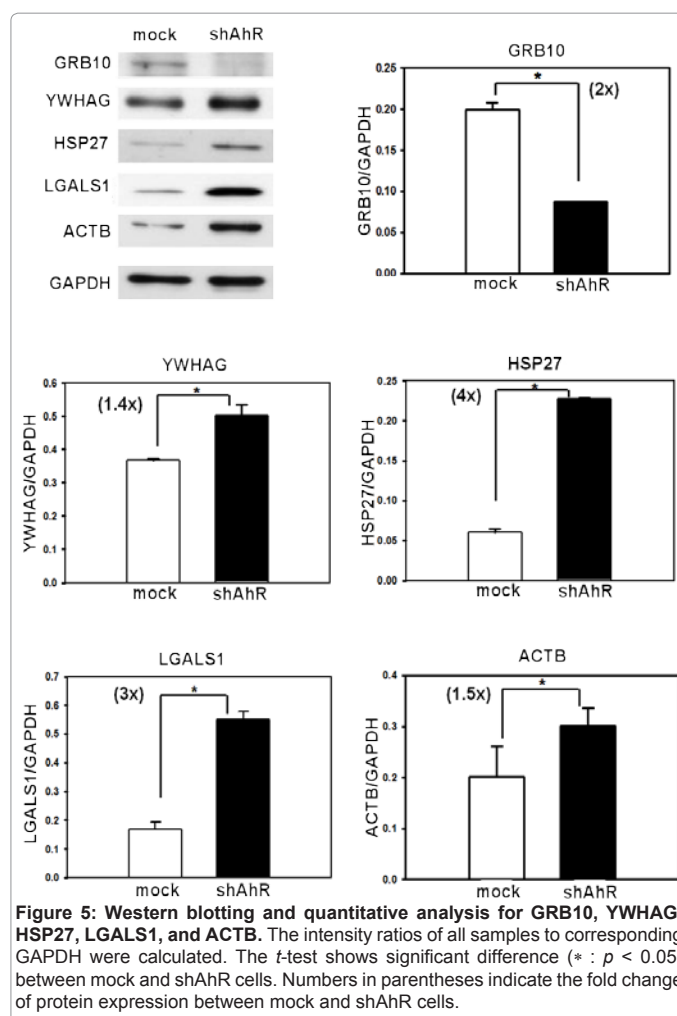
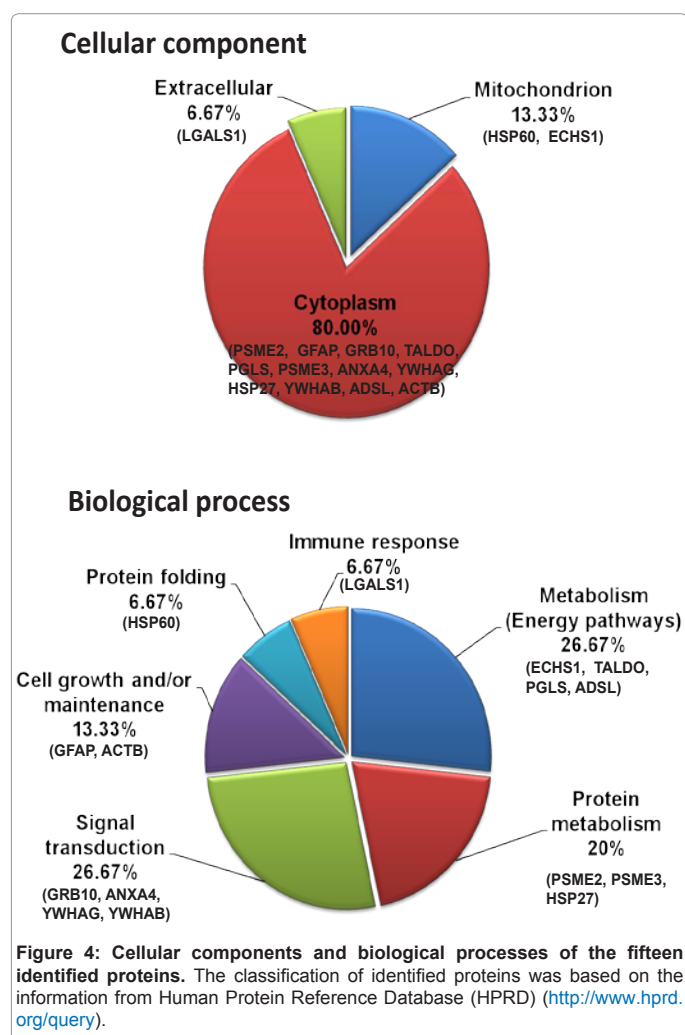


**Figure 3: Detailed 2DE images of differentially produced proteins in mock and shAhR cells.** The fifteen proteins were identified from the seventeen protein spots. There were two proteins, HSP27 and ACTB, identified with two group spots (group 1 of G and H; group 2 of L and M). Arrows on the map indicated protein spots with differential expression in mock and shAhR cells.

PSME3, ANXA4, YWHAG, HSP27 and ACTB interacted with the four apoptosis-associated proteins.

## Discussion

For the first time, proteomic approach is applied to the comprehensive study of the effect of AhR-deficient in human lung cells with TCDD treatment. Previously, we have investigated into the PCDD/F levels including TCDD in environmental victuals and human serum and therefore we very interest the molecular mechanisms to lead to carcinogenicity, teratogenicity, immune dysfunction and endocrine disruption. However, several previous studies have demonstrated that the numerous pulmonary diseases are induced by TCDD exposure [6,34]. In fact, the mechanisms of TCDD-mediated AhR signaling pathway is still poor understood, particularly in lung cancer. In present study, to apply the comparative proteomic technique of 2DE to compare between normal and reduced AhR expression in the same human lung cell line, H1355. The powerful tool of 2DE for visualizing and screening large-scale numerous proteins was utilized to separate the proteins of TCDD-treated control (mock) and AhR-deficient (shAhR) lung adenocarcinoma cells. After image analysis, seventeen spots had the significantly differential expression level and therefore the protein identification was performed using the LC-MS/MS analysis. The fifteen identified proteins implicate the possibility of



participation in the some mechanisms of TCDD-mediated AhR. In further analysis, the protein-protein interactive network of identified proteins is generated with an integrated software suite. It is relevant that twelve of fifteen identified proteins involved in apoptosis pathway, including Galectin-1, ACTB, GFAP, 6PGL, GRB10, PSME3, 14-3-3 beta/alpha, Annexin IV, 14-3-3 gamma, HSP60, PSME2 and HSP27 (Figure 6). The five apoptosis-associated proteins were validated by Western blotting and the quantitative results concurred with that from 2DE gel image analysis. This suggests that twelve apoptosis-associated proteins play a part in TCDD-mediated changes of AhR expression in lung adenocarcinoma.

We provide evidence that apoptosis pathway may be as the main respondent biological process between the control and AhR-deficient lung cancer cells by TCDD exposure. Apoptosis also known as programmed cell death is an important and complicated process in cell to be regulated by a diverse range of molecular signaling systems. The belief is presented that normal cells lose control for apoptosis to become abnormal cells and then contribute to cancer initiation [35]. A number of contradictory studies report that TCDD-mediated AhR can elicit apoptosis in some tissues and cell types and inhibit it in others [36]. In the present study, four significant apoptotic factors of caspase-3, p53, MDM2 and Akt/PKB have been demonstrated that the complex interactions play important roles in the pro-apoptotic and



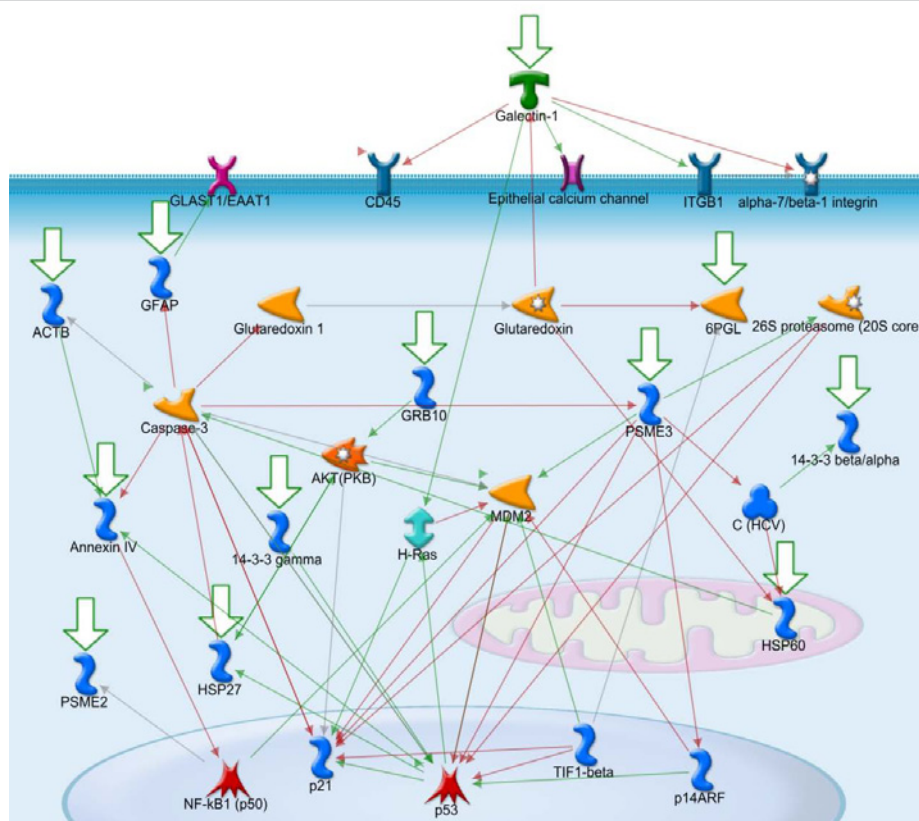
anti-apoptotic signals [37-39]. One of the understanding pathways is that Akt/PKB kinase phosphorylates MDM2, which is an E3 ubiquitin ligase and increases its activity and then MDM2 ubiquitinates p53 to lead to p53 proteasomal degradation [40-41]. As a feedback loop, p53 targets to MDM2 gene promoter and induces MDM2 expression [42]. In a recent study, TCDD can increase MDM2 protein level and inhibit p53 response to influence the liver carcinogenesis [43]. It is also verified that TCDD increases Akt phosphorylation by inducing PI3K activity to lead to suppression of cell death [44]. In Figure 6, the three identified proteins of GRB10, PSME3 and HSP27 interact to the Akt/PKB-MDM2-p53 signaling axis following the alteration of TCDD-mediated AhR pathway in lung adenocarcinoma cells.

On the other hand, a largest proportion of identified proteins interact with caspase-3 in the interactive network. Caspase-3, which is a cysteine-aspartic acid protease, is indispensable for apoptosis in mammals, particularly in oxidative stress-induced [45]. According to our previous study, TCDD induces oxidative stress in human breast carcinoma cell lines [9]. We suggest that TCDD may induce oxidative stress to modulate the caspase-3-mediated apoptosis in human lung cells. In chondrocytes, the other study also demonstrates that TCDD induces ROS-mediated caspase-3 activity to modulate apoptosis process [46]. Consequently, our study unambiguously indicated that the most of identified proteins, which are the differential expression level between the TCDD-treated mock and shAhR cells, are involved in two

major apoptosis-related signaling pathways of Akt/PKB-MDM2-p53 and caspase-3-mediated axes. Furthermore, some of identified proteins in Figure 6 have been demonstrated to be regulated by TCDD exposure such as HSP60, GFAP, HSP27 and LGALS1 in different investigations [30, 47-49]. Herein, these proteins may respond to apoptosis via TCDD-mediated AhR pathway. There are still many potential targets remaining to be investigated.

In addition, we utilized human cancer pathway microarray assay to investigate the gene expression changes between control and AhR-deficient cells by TCDD exposure (Supplementary Table 1). In the results of differential gene expression levels, seven apoptosis-associated genes were significant changes (shAhR/mock ratio 3 or 0.33), including, APAF1, BAD, BCL1L2, CFLAR, NFKB1, PIK3CB and FAS. The seven genes were a relatively high percentage in 20 significant changed genes. These data combined with proteomics results would strongly support the hypothesis that TCDD-mediated AhR in H1355 cells takes place mainly through an apoptotic process.

In our present result, the higher expression level of GRB10 in mock cells compared to shAhR cells is considered to quantification of 2DE images and Western blotting (Figure 3 & 5). For the first time, it is demonstrated that GRB10 is reduced by TCDD treatment in AhR-deficient lung adenocarcinoma cell line. The molecular function of GRB10 is as an adaptor protein that interacts with some tyrosine kinase receptors and signaling molecules, including EGFR, Ret and IGF-R



**Figure 6: The interaction network analysis of proteins of altered expression level between mock and shAhR cells.** The interactive map was illustrated by the MapEditor program appended to MetaCore. Proteins identified in this study (Galectin-1 (LGALS1), ACTB, GFAP, 6PGL (PGLS), GRB10, PSME3, 14-3-3 beta/alpha (YWHAB), Annexin IV (ANXA4), 14-3-3 gamma (YWHAG), HSP60, PSME2 and HSP27) were marked with a white arrow on top. The symbols and links shown on the network illustration include: generic binding protein (blue), receptor ligand (green), transporter (orange), generic receptor (yellow), generic channel (red), generic enzyme (orange), generic protease (yellow), protein kinase (orange), protein phosphatase (orange), Ras- superfamily (green), protein (blue), transcription factor (red), activation (green), inhibition (red), and unspecified (grey).

[50]. The SH2 domain in GRB10 is as a trigger for phosphorylation of tyrosine kinase that has been reported to regulate cell proliferation and apoptosis and also implicates in tumor progression [51]. Furthermore, GRB10, which is as a positive regulator, interacts with a synergistic factor of c-kit to activate Akt/PKB [52]. Therefore, these evidences reveal that GRB10 is involved in the mechanism of apoptosis. It is further studied that GRB10 possesses an anti-apoptotic activity through interaction with Raf-1 to contribute in cell survival underlying tumorigenesis [53-54]. Other researchers have indicated that TCDD treatment in B cell leads to alteration of phosphorylation level of kinases such as Akt/PKB, ERK and JNK [55]. Nevertheless, the mechanism of regulation of Akt/PKB in TCDD-mediated AhR pathway is still unclear.

Taken together, we hypothesize that GRB10 is regulated by the complex of TCDD-liganded AhR involved in the apoptosis pathway of Akt/PKB-MDM2-p53 signaling axis. In detail, we integrate with our previous and present study to suggest that GRB10-mediated apoptosis leads to the reduced AhR expression in lung adenocarcinoma cell to decrease intracellular oxidative stress and suppress cell growth [26]. To verify the hypothesis, the expression level of GRB10 compared between normal AhR-expressed (mock) and AhR-deficient (shAhR) cells are examined by Western blotting (Figure 5). The GRB10 expression in shAhR cells is significantly decreased two-fold ( $p < 0.05$ ), compared to mock cells. Therefore, our data demonstrate that disrupting AhR expression with TCDD treatment reduces the expression of GRB10.

In conclusion, we have provided strategy that protein expression in comparative proteomic approach of TCDD toxicity. The strategy has often been applied that only one gene over-expressed or silenced in the same cell line decreases the variables and perturbation in proteomic study. We apply the lung adenocarcinoma cell line with reduction of AhR expression to recognize the differential expression of proteins and analyze those involved in particular biological mechanisms. In present study, we find that the most of identified proteins are involved in apoptosis pathway. GRB10 has a critical role to regulate the cell apoptosis and proliferation by TCDD-liganded AhR in lung adenocarcinoma cell. It may lead to be as a potential target to prevent the development of lung adenocarcinoma.

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