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Molecules and Cells

Identification of EBP50 as a Specific Biomarker for Carcinogens Via the Analysis of Mouse Lymphoma Cellular Proteome

Yoen Jung Lee, In-Kwon Choi, Yhun Yhong Sheen[1], Sue Nie Park[2], and Ho Jeong Kwon*

To identify specific biomarkers generated upon exposure of L5178Y mouse lymphoma cells to carcinogens, 2-DE and MALDI-TOF MS analysis were conducted using the cellular proteome of L5178Y cells that had been treated with the known carcinogens, 1,2-dibromoethane and *O*-nitrotoluene and the noncarcinogens, emodin and D-mannitol. Eight protein spots that showed a greater than 1.5-fold increase or decrease in intensity following carcinogen treatment compared with treatment with noncarcinogens were selected. Of the identified proteins, we focused on the candidate biomarker ERM-binding phosphoprotein 50 (EBP50), the expression of which was specifically increased in response to treatment with the carcinogens. The expression level of EBP50 was determined by western analysis using polyclonal rabbit anti-EBP50 antibody. Further, the expression level of EBP50 was increased in cells treated with seven additional carcinogens, verifying that EBP50 could serve as a specific biomarker for carcinogens.

INTRODUCTION

Carcinogens are substances capable of causing cancer in humans or animals. If a substance is known to promote, but not necessarily cause cancer, it can be considered as a carcinogen (Perera and Weinstein, 1982). Some carcinogens act on DNA directly and some lead to cancer in other ways that are not involved in the direct interaction with DNA. For example, a carcinogen may cause cells to divide more rapidly than normal, which can increase the chance that changes will occur in DNA. Evaluation of the carcinogenic potential of a new compound has been costly and time-consuming. Therefore, a rapid assay capable of predicting the carcinogenicity of compounds would facilitate the process of drug development (Bandara and Kennedy, 2002).

Toxicoproteomics is a field of research that focuses on the identification of critical proteins and pathways in biological systems that are affected by and respond to adverse chemicals and environmental exposure using global protein expression technologies. Proteome profiling permits the screening and selection of candidate biomarkers based on differential expression in experimental and control cells. Selected proteins can be validated using molecular and cell biological tools. Biomarkers based on proteomics analysis can be applied for immunohistochemistry and evaluation of toxicity and safety of compounds and used for reducing animal testing, a common method of safety evaluation.

In this study we exposed L5178Y mouse lymphoma cells to two carcinogens, 1,2-dibromoethane and *O*-nitrotoluene, and two noncarciongens, emodin and D-mannitol, in an effort to identify specific biomarkers for carcinogens. L5178Y mouse lymphoma cells are recommended for use in chemical testing because of their well-characterized cellular response and high sensitivity to DNA damage as a result of loss of cell cycle control (Clark et al., 1998). One of the carcinogens used in this study, 1,2-dibromoethane has been used as a pesticide and gasoline additive and possesses a potent risk to humans exposed environmentally or in an industrial situation (Hawkins et al., 1998). This compound is a well-known carcinogen and causes liver and kidney toxicity (Humphreys et al., 1999) and induces skin, lung, and stomach cancer (Letz et al., 1984; Olson et al., 1973; Rannug, 1980). *O*-nitrotoluene is used to synthesize agri-cultural and rubber products and causes hemangiosarcoma, cecum cancer, and hepatocellular carcinoma (Sills et al., 2004). Of the two noncarcinogens, emodin is used as an anti-viral and a liver-protective agent (Arosio et al., 2000). In addition, D-mannitol is used as an osmotic laxative.

We compared the consequence of exposure of L5178Y mouse lymphoma cells to the two carcinogens and two noncarcinogens using 2-DE and MALDI-TOF/TOF MS analysis and identified ERM-binding phosphoprotein 50 (EBP50) as a specific biomarker for carcinogens. EBP50 was further validated by western analysis and examination of its expression level following exposure of cells to an additional seven carcinogens. Here, we report for the first time that EBP50 could serve as a specific biomarker for monitoring carcinogenic potential of a compound in L5178Y mouse lymphoma cells.

Department of Biotechnology, Translational Research Center for Protein Function Control, College of Life Science and Biotechnology, Yonsei University, Seoul 120-749, Korea, [1]College of pharmacy, Ewha Womans University, Seoul 120-750, Korea, [2]Hazardous Substances Analysis Division at Seoul Regional FDA, Korea Food and Drug Administration, Seoul 158-050, Korea
*Correspondence: kwonhj@yonsei.ac.kr

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MATERIALS AND METHODS

Materials

1,2-Dibromoethane, *O*-nitrotoluene, glycidol, 8-hydroxyquinoline, emodin, methylcarbamate, D-mannitol, 1,2-dichlorobenzene, and 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD) were purchased from Accustandard Inc. (USA). Diethylstilbestrol was purchased from Fluka (Switzerland). Caprolactam and bisphenol A were purchased from Aldrich (USA). 1,4-Dioxane, tetrachloroethylene, acetonitrile, and L-ascorbic acid were purchased from Sigma-Aldrich (Italy). Urethane and chlorpheniramine maleate were purchased from Sigma (USA). RPMI 1640, antibiotics, and horse serum were purchased from Invitrogen (USA). PVDF membrane and enhanced chemiluminescence (ECL) kit were purchased from Millipore (USA) and GE Healthcare (UK), respectively. Rabbit anti-EBP50 polyclonal antibodies and mouse anti-β-actin monoclonal antibodies were obtained from Abcam (UK). HRP-conjugated sheep anti-mouse and donkey anti-rabbit were purchased from GE Healthcare (UK).

Cell culture and sample preparation

L5178Y mouse lymphoma cells were grown in RPMI 1640 supplemented with 10% (v/v) heat-inactivated horse serum and 1% antibiotics at 37°C in a humidified atmosphere of 5% CO_2/95% air. Cultured cells were seeded in T75 flasks under the conditions described above. After a 2 h incubation, test compounds or solvent (DMSO only) were added to the cultures followed by incubation for 2 h. Treated cells were harvested and test compounds were removed by centrifugation at 3,000 rpm in RPMI. Cells were reseeded in T75 flasks and incubated for 22 h, followed by harvesting and sample preparation.

2-Dimensional electrophoresis (2-DE)

2-DE was conducted as described previously (Cho et al., 2005). Briefly, cell pellet were suspended in sample buffer containing 7 M urea, 2 M thiourea, 4.5% CHAPS, 100 mM DTE, 40 mM Tris, pH 8.8. Suspensions were sonicated for approximately 30 sec and centrifuged at 100,000 × *g* for 45 min. One milligram of total protein was used for each electrophoresis. Aliquots of each proteins in sample buffer were applied onto immobilized pH 3 to 10 nonlinear gradient strips (immobilized pH-gradient strips; Amersham Pharmacia Biotech, Sweden). Isoelectric focusing was carried out in the first dimension at 80,000 Vh and molecular weight determination was carried out in the second dimension in 9-17% linear gradient polyacrylamide gels at a constant current of 40 mA per gel for approximately 5 h. Following electrophoresis gels were fixed in 40% methanol/5% phosphoric acid for 1 h. The 2-DE gels were stained with Coomassie Brilliant Blue G-250 for 12 h and destained with distilled water. The gels were scanned with a GS-710 imaging densitometer (Bio-Rad) and converted to electronic files. Images were analyzed with the Image Master Platinum 5 program (GE Healthcare).

Identification of proteins by MS

For 2-D gel mapping of the compound-treated proteome, spots were identified by peptide mass fingerprinting (Lu et al., 2009). Protein spots excised from 2-DE gels were destained, reduced, alkylated, and then digested with trypsin. Trypsin-digested peptides were desalted using a porous resin and then purified. Trypsin digestion and desalting processes were conducted as previously described (Cho et al., 2005). Peptides were prepared for MALDI-TOF MS by mixing with matrix (alpha-cyano-4-hydroxy cinnamic acid, CHCA) and 2% formic acid in 70% acetonitrile and droplets were allowed to dry on the MALDI plate (Opti-TOF™ 384 well Insert, Applied Biosystems). MALDI-TOF MS was performed on a 4800 MALDI-TOF/TOF™ Analyzer (Applied Biosystems) and the mass spectra were obtained in the reflectron mode with an accelerating voltage of 20 kV and sum from 500 laser pulses and calibrated using the 4700 calibration mixture (Applied Biosystems). Data Explorer 4.4 (PerSeptive Biosystems) was used for data acquisition and extraction of the monoisotopic masses. NCBInr human protein database (http://www.ncbi.nlm.nih.gov) searching was performed with the MASCOT search engine (http://www.matrixscience.com). Database search criteria were, taxonomy, *Mus musculus,* fixed modification, No, variable modification, oxidized (+16) at methionine residues and carboxyamidomethylated (+57) at cysteine residues, maximum allowed missed cleavage, 1, MS tolerance, 100 ppm. Only peptides resulting from trypsin digests were considered.

Western analysis

L5178Y mouse lymphoma cells were seeded at 1×10^6 cells/ml in 6-well plates and treated with test compounds for 2 h. Cell lysates were prepared and separated by 10% SDS-PAGE followed by transfer to PVDF membranes. Membranes were blocked in 3% skim milk in 1X Tris-buffered saline containing 0.1% Tween-20 (TBS-T) for 1 h at room temperature. Blocked membranes were incubated overnight at 4°C with the primary antibodies polyclonal rabbit anti-EBP50 and monoclonal mouse anti-β-actin. The next day membranes were washed with TBS-T and incubated for 1 h with either HRP-conjugated anti-mouse or anti-rabbit secondary antibody. Immunoreactivity was assessed by ECL.

Statistical analysis

Results were expressed as the mean ± standard deviation. Student's *t*-test was used to determine statistical significance between control and experimental groups. $P < 0.05$ was considered statistically significant.

RESULTS

Analysis of the proteome of L5178Y mouse lymphoma cells treated with carcinogens and noncarcinogens

To analyze the mouse lymphoma cellular proteome using 2-DE, L5178Y mouse lymphoma cells were treated with the two carcinogens, 1,2-dibromoethane (100 μg/ml) and *O*-nitrotoluene (200 μg/ml), and the two noncarcinogens, emodin (30 μg/ml) and D-mannitol (5,000 μg/ml). Conditions for treatment of compounds were determined by cytotoxicity test in L5178Y mouse lymphoma cells (Go and Sheen, 2008). Briefly, L5178Y mouse lymphoma cells were seeded and treated with three doses of test compounds for 2 h. 80% of cell viability was considered as a standard and cells were further incubated in normal media. After 22 h incubation, doses which showed more than 60% of cell viability were determined as the effective concentrations.

Proteome from compound-treated cells was separated by 2-DE and image analysis was carried out. Protein spots were categorized in two groups, carcinogen- and noncarcinogen-treated samples and representative patterns of each gel images were compared between the two groups. Eight protein spots that showed greater than 1.5-fold variation in carcinogen-treated samples and no change in the noncarcinogen-treated samples were selected for further characterization (Fig. 1). Selected protein spots were identified by MALDI-TOF MS analysis and identified proteins are listed in Table 1. From the result, highly abundant and common proteins, for example

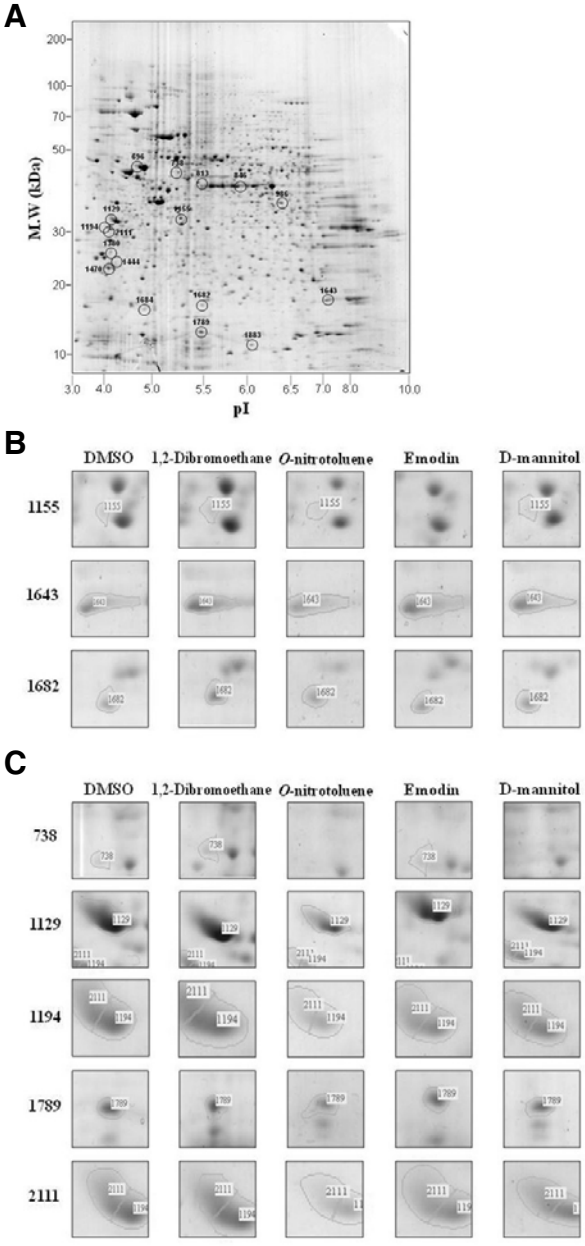


Fig. 1. The 2-D gel of proteins isolated from L5178Y mouse lymphoma cells treated with test compounds. (A) Sample gel image of solvent control-treated proteome profile. Proteins were separated on the basis of pI (*x*-axis) and molecular mass (*y*-axis). Spots were visualized by Coomassie blue staining. (B) Enlarged images of up-regulated protein spots. (C) Enlarged images of down-regulated protein spots. Selected spots were defined as altered and then were identified by MALDI-TOF MS analysis.

immunoglobulin and keratin, were eliminated on the selection of candidate biomarker.

One of the upregulated proteins, EBP50 was selected to validate the result of proteome analysis, since the expression level of EBP50 can be linked with carcinogenic potential. EBP50 is a cytoplasmic protein with two postsynaptic density-95/Disk-large/ZO-1 homologous (PDZ) domains and a β-catenin-associating domain. EBP50 can interact with β-catenin through its

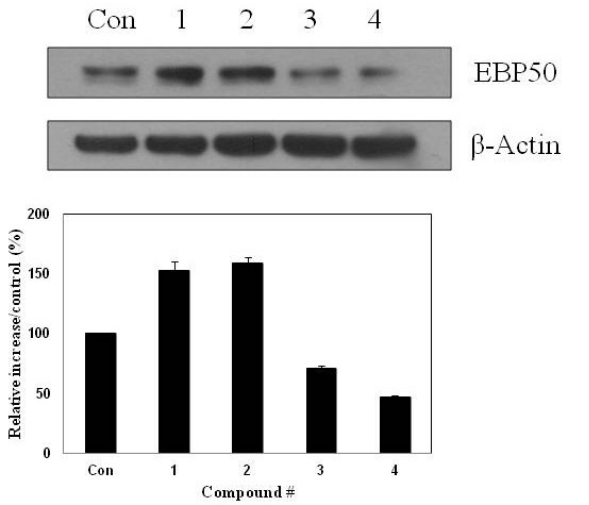


Fig. 2. Western analysis of EBP50 protein expression level following treatments. Con: solvent control (DMSO), 1 to 4: 1,2-dibromoethane, *O*-nitrotoluene, emodin, and D-mannitol, respectively. L5178Y mouse lymphoma cells were treated with each compound at different effective concentrations for 2 h. β-actin was used as a loading control.

carboxyl-PDZ domain *in vitro* and *in vivo* (Shibata et al., 2003). In addition, correlation between EBP50 and cancer has been reported in human hepatocellular carcinoma (Shibata et al., 2003) and breast cancer (Song et al., 2007).

Expression level of EBP50 increases with treatment of carcinogens but not with noncarcinogens

To determine the expression level of EBP50, L5178Y mouse lymphoma cells were treated with each compounds, 1,2-dibromoethane, *O*-nitrotoluene, emodin, D-mannitol (100, 200, 30, and 5000 μg/ml, respectively) and solvent control (DMSO), as described in "Materials and Methods", followed by western analysis of the cell lysates. Increases of 150% and 160% were observed in 1,2-dibromoethane- and *O*-nitrotoluene-treated samples, respectively, compared to the solvent control (Fig. 2). In contrast, the expression levels of EBP50 in emodin- and D-mannitol-treated samples were not showed increasing patterns. Since treatment of cells with two different carcinogens caused a greater than 150% increase in EBP50 expression, we considered EBP50 a candidate biomarker for detection of carcinogens and further investigation was conducted with the protein.

EBP50 can be a specific biomarker for carcinogens

To further verify the potential of EBP50 as a specific biomarker for carcinogens, we determined its expression level in L5178Y mouse lymphoma cells in response to other well-known carcinogens. Seven carcinogens were additionally selected on the basis of literature information; these were glycidol; diethylstilbestrol; urethane; methylcarbamate; 1,4-dioxane, tetrachloroethylene; and 2,3,7,8-tetrachlorodibenzo-*p*-dioxine (TCDD). The carcinogenic properties of these compounds have been reported in previous studies (Biswas et al., 2008; Gladek and Liehr, 1989; Hübner et al., 1997; Kwon et al., 2007; Paulu et al., 1999; Stickney et al., 2003; Yamamoto et al., 2001). Also seven non-carcinogens were selected for testing; these were 8-hydroxyquinoline; acetonitrile; L-ascorbic acid; 1,2-dichloroben-zene; caprolactam; bisphenol A; and chlorpheniramine maleate (Hellbom, 2006; Kurt et al., 1991; McFee and Lowe, 1989; McFee,

Table 1. Proteins identified by 2-DE and MALDI-TOF MS analysis

	Spot no.	Protein name	NCBI accession number	Fold difference (carcinogen/ control)	Fold difference (noncarcinogen/ control)	MW (Da)/pI	No. of peptides matched	Sequence coverage (%)
Up-regulated	1155	Solute carrier family 9, isoform 3 regulator 1 (EBP50)	gi|148702516	2.0	0.5	31623/6.60	7	38
	1643	Partitioning-defective protein 3 homolog isoform 2	gi|61888842	1.6	1.1	66568/8.50	11	19
	1682	Immunoglobulin heavy chain variable region	gi|11692656	1.7	1.0	10875/9.13	4	66
Down-regulated	738	TFII-I repeat domain-containing protein 3 beta 7	gi|19908490	0.5	1.0	123402/6.78	10	10
	1129	Immunoglobulin heavy chain variable region	gi|66990025	0.6	0.8	12510/8.90	4	61
	1194	Fibroblast growth factor receptor-like 1 isoform 1	gi|16905101	0.5	0.8	56977/9.69	7	17
	1789	RAD18 homolog (*S. cerevisiae*), isoform CRA_c	gi|21362877	0.6	1.1	63114/6.26	12	34
	2111	Keratin complex 1, acidic, gene 10	gi|112983636	0.6	1.1	57007/5.00	11	22

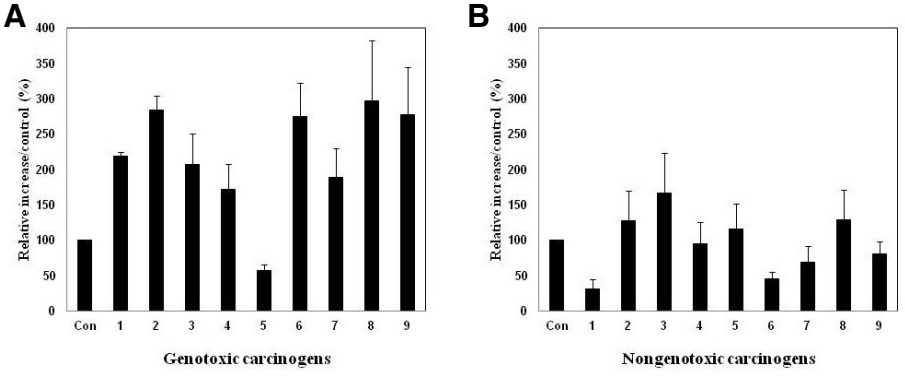


Fig. 3. Specific expression of EBP50 by carcinogen treatments. L5178Y mouse lymphoma cells were treated with compounds for 2 h. The effective concentration of each compound is shown in Table 2 and 3. (A) Treatment of carcinogens. Con: solvent control (DMSO), 1 to 9: 1,2-dibromoethane, glycidol, diethylstilbestrol, urethane, methylcarbamate, *O*-nitrotoluene, 1,4-dioxane, tetrachloroethylene, and TCDD, respectively. (B) Treatment of noncarcinogens. Con: solvent control (DMSO), 1 to 9: 8-hydroxyquinoline, emodin, acetonitrile, L-ascorbic acid, D-mannitol, 1,2-dichlorobenzene, caprol-actam, bisphenol A, and chlorpheniramine maleate, respectively.

1989; Shamberger, 1984; Steinmetz et al., 1997). L5178Y mouse lymphoma cells were exposed to the carcinogens and noncarcinogens at their effective concentrations (Tables 2 and 3) as determined by cytotoxicity test (Go and Sheen, 2008).

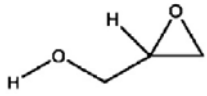
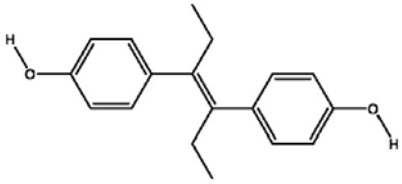
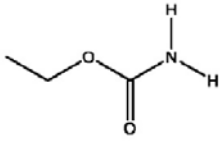
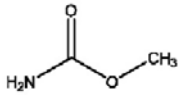
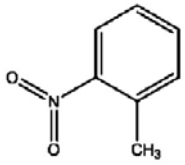
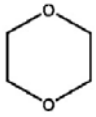
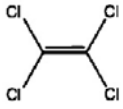
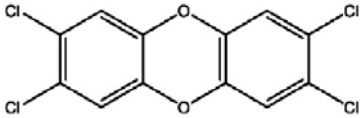
The specificity of EBP50 was calculated based on the results of western analysis (Fig. 3). The expression level of EBP50 compared to solvent control was quantitatively measured by using the Image J program. The specificity is expressed as percentage and is the ratio of the number of test compounds which showed a greater than 150% increase in expression level to the total number of compounds. Of nine carcinogens, eight carcinogens (1,2-dibromoethane, glycidol, diethylstilbestrol, urethane, *O*-nitrotoluene, 1,4-dioxane, tetrachloroethylene, TCDD) exhibited a greater than 150% (± 0.36, n = 3) increase in expression level of EBP50. This indicates that the specificity of EBP50 for the carcinogens is 88.9%. Among nine carcinogens, only methylcarbamate-treated sample was showed decrease (56.9%) in EBP50 expression. With regard to the noncarcinogens tested in this study, the specificity of EBP50 is 11.1% since only acetonitrile-treated sample showed a greater than 150% (± 0.27, n = 2) increase in expression level among nine noncarcinogens. These results suggest that EBP50 could serve as a specific biomarker for detection of carcinogenicity of the given compounds.

DISCUSSION

The aim of this study was to identify and validate a specific biomarker for carcinogens. Rapid prediction of the carcinogenic potential of new compounds by using specific biomarkers would be a highly effective tool in drug development (Bandara and Kennedy, 2002), especially reducing animal testing. To identify the specific biomarker for carcinogens, proteomics-based approaches including 2-DE and MALDI-TOF MS analyses were used in this study.

L5178Y mouse lymphoma cell line used in this study is a

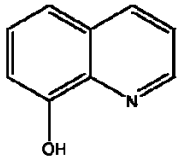
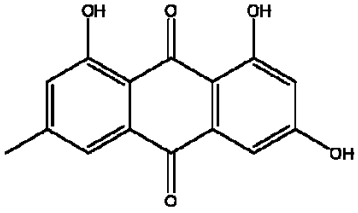
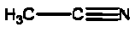
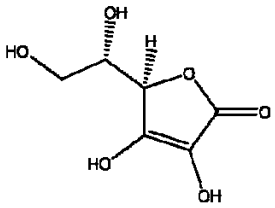
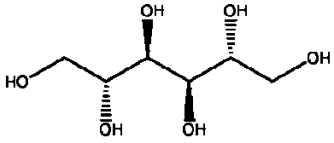
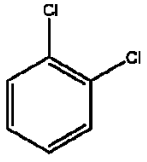
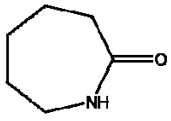
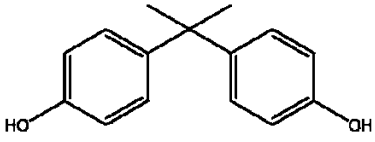
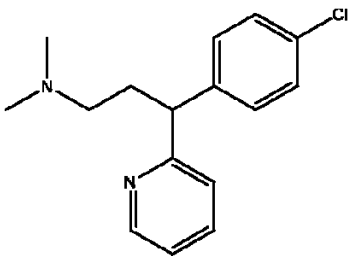
Table 2. Carcinogen structures and concentrations for cell treatment

Carcinogen	Chemical structure	Concentration (μg/ml)
1,2-Dibromoethane		100
Glycidol		400
Diethylstilbestrol		15
Urethane		5,000
Methylcarbamate		5,000
O-nitrotoluene		200
1,4-Dioxane		4000
Tetrachloroethylene		100
TCDD		10

commonly used cell line for toxicity test because this cell line is highly sensitive to DNA damage by the mutation of thimidine kinase gene. L5178Y mouse lymphoma cells were treated with two carcinogens and two noncarcinogens and their cellular proteomes were analyzed by 2-DE. Eight protein spots showed greater than 1.5-fold increase or decrease patterns in the case of carcinogen-treated were chosen (Fig. 1). These proteins were then identified by MALDI-TOF MS analysis (Table 1).

Up and downregulated proteins identified in carcinogen-treated samples in this study had been shown to be related to cancer in previous reports. One of these proteins, EBP50, showed a pattern of increased expression in carcinogen-treated samples. Overexpression of EBP50 was detected in human hepatocellular carcinoma (Shibata et al., 2003) and this protein is known to be a potent marker of invasiveness of breast cancer (Song et al., 2007). In contrast, fibroblast growth factor receptor-like 1 isoform 1 was downregulated in carcinogen-treated samples; loss-of-function mutations of fibroblast growth factor-receptor-like 1 isoform 1 have been reported in human melanoma (Katoh, 2009). In light of these studies, selected protein

Table 3. Noncarcinogen structures and concentrations for cell treatment

Noncarcinogen	Chemical structure	Concentration (μg/ml)
8-Hydroxyquinoline		100
Emodin		30
Acetonitrile		5000
L-ascorbic acid		2500
D-mannitol		5000
1,2-Dichlorobenzene		50
Caprolactam		4000
Bisphenol A		50
Chlorpheniramine maleate		70

spots can be further developed as biomarkers for detection of carcinogenic potential of a compound. At this point, we selected EBP50 as a candidate biomarker for carcinogens to validate the results from proteome analysis.

To verify the expression level of EBP50, cells were treated with test compounds (1,2-dibromoethane, *O*-nitrotoluene, emodin, D-mannitol) and western analysis was followed. Carcinogen-treated samples showed increase patterns in EBP50 expression whereas noncarcinogen-treated samples showed decrease patterns or no change in expression of EBP50 (Fig. 2). Since upregulated EBP50 expression was confirmed in carcinogen-treated samples, EBP50 was selected for further investigation.

To further investigate the potential of EBP50 as a specific biomarker for carcinogens, we determined the expression of EBP50 in L5178Y mouse lymphoma cells following treatment with nine carcinogens and nine noncarcinogens at their effective concentrations (Tables 2 and 3). Western analysis showed that treatment with eight of the carcinogens resulted in increased pattern; only one of the noncarcinogen-treated sample showed an increase pattern of EBP50 (Fig. 3). Based on these results, the specificity of EBP50 was quantitatively calculated; it showed 88.9% specificity for carcinogens and 11.1% for noncarcinogens, respectively. In addition, the carcinogenic potential of test compounds with their effective concentrations was not detected in normal NIH3T3 mouse fibroblasts. These results suggest that EBP50 could serve as a specific biomarker for detection of carcinogenicity of a given compound in L5178Y mouse lymphoma cells.

Collectively, EBP50 is identified and validated as a specific biomarker for carcinogens based on differences in the patterns of its expression level between carcinogen-treated and noncarcinogen-treated L5178Y mouse lymphoma cells using proteomics analysis. As a specific biomarker for carcinogens, EBP50 can be applied for tissue biomarker by immunohistochemistry and used for safety/toxicity evaluation of a given compound prior to animal testing for prediction of compound carcinogenicity.

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