RESPONDING PROTEINS FOR HACAT CELLS AGAINST 2,4-DINITROBENZENE SULFONIC ACID STIMULATION–A PROTEOMIC STUDY

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ABSTRACT

2,4-dinitrobenzene sulfonic acid (DNBS) contributes to the incidences of allergic dermatitis, inflammatory enteritis and colon cancer. In this study, the responding proteins of human keratinocyte HaCaT cells against DNBS stimulation were separated by two-dimensional difference in gel electrophoresis (2DDIGE), quantified by DeCyder software, post-stained by Deep Purple. And the tryptic digested proteins were identified by high performance liquid chromatography combined to nano-electro-spray ionization tandem mass spectrometry (HPLC-nESI-MS/MS) or matrix-assisted laser desorption ionization (MALDI) MS. Six most up-regulated proteins in HaCaT against DNBS stimulation were chromosome X ORF 26 (Cxor26), co-chaperone P23 (PTGES3), calmodulin (CALM3), interferon-gamma inducing factor precursor (IL-1β), smooth muscle/non-muscle myosin alkali light chain (MYL6) and breakpoint cluster region protein 1 (BANF1). Two most down-regulated proteins were elongin B isofrom alpha (TCEB2) and ribosomal protein L23 (RPL23). Their level changes were further validated by Western blotting and qRT-PCR assays. DNBS affects HaCaT proteome. Most of identified protein candidates were reported for the first time to be involved in skin cell damage to chemical stimulation. This work contributes to the understandings of risk assessment and toxicological mechanism of skin diseases caused by chemical carcinogens.

Keywords: DNBS, HaCaT, DIGE, proteomics.

INTRODUCTION

Skin is a target organ for external offenders, such as UV irradiations, ionizing radiations and chemical toxicities (Petrova et al., 2011; Vicentini and Simi, 2008; Yanti and Hwang, 2010). Skin disease has become a major disease seriously threatening public health (Damian et al., 2008; Hopper et al., 2009; Housman et al., 2003). The new cases of skin cancers diagnosed each year is equivalent to the incidence of malignancies happen in all other organs combined (Katiyar, 2007). The constant increases in life expectancy as well as changes in environmental conditions, dietary habits and lifestyle are contributed to the development of skin diseases (Damian et al., 2008; Hensbergen et al., 2005; Hopper et al., 2009; Housman et al., 2003; Katiyar, 2007).

The widespread use and exposure of chemical carcinogens is one of the major causes of skin diseases (Harper, 2004; Housman et al., 2003). 2,4-dinitrobenzene sulfonic acid (DNBS), a member of nitrosulfonic acids, is widely used in the industries of faded plating, dye and paint. It can cause allergic dermatitis, inflammatory enteritis, colon cancer and other diseases (Guzzocrea et al., 2001; Rijnierse et al., 2006). The incidences of skin cancer, allergic dermatitis and stomach cancer significantly increase for field workers due to long-term exposure to chemical allergens (Guzzocrea et al., 2001; Harper, 2004). The study on skin cell damage and skin diseases caused by chemical carcinogens at protein level is poorly performed. The molecular mechanisms of skin damages and diseases induced by DNBS stimulation are unknown.

Proteomics has emerged as a promising tool to target differentially expressed proteins, to screen novel targets and create possible therapeutic interventions for human diseases. HaCaT cells are immortalized human keratinocyte cell owing similar differentiation properties with nice proliferation ability and high stability and have been used as the ideal experimental replacements for normal keratinocytes. In current work, two-dimensional difference in gel electrophoresis (2D DIGE) was performed to screen the potential protein targets in HaCaT responding to DNBS stimulation. Deep Purple fluorescent staining was performed for post-electrophoresis gel. Most protein candidates were revealed for the first time to be involved in skin cell damage from chemical carcinogen. This work might provide new insight into the understanding of risk assessment and toxicological mechanism of skin diseases caused by chemical carcinogens.

MATERIALS AND METHODS

Chemicals and instruments: RPMI 1640 and FBS were from Gibco (US). Cy2, Cy3, Cy5. Deep Purple, pH 3-10 NL IPG strip (24cm), 1,4-dithiothreitol (DTT),
Acrylamide (Acr), carrier ampholyte, lysine, sodium dodecyl sulfate (SDS), N,N'-methylenebisacrylamide (Bis), 2D-Quant protein assay kit, thiourea and urea were obtained from GE Healthcare (US). Ammonia bicarbonate (ABC), ammonium persulfate (AP), acetonitrile (ACN), 3-[(3-cholamidopropyl)dimethylammonio]-1-propane sulfonate (CHAPS),DNBS, formic acid (FA), iodoacetamide (IAM), and cell lysis solution were from Sigma (US). Modified trypsin was from Promega (Madison, US). All other chemicals were of analytical grade.

Etten IPG phor II, DeCyder 6.5, Typhoon 9400 scanner and Etten Spot Picker were from GE (US).C18 column (150 × 0.075mm) was from Dionex (US). LTQ mass spectrometer and TurboSequest Bioworks were from Thermo Finnigan (US). ProOTOF 2000 mass spectrometer was from Perkins Elmer (US).

Cell culture: HaCaT cells were incubated in 90% RPMI1640 supplemented with penicillin/streptomycin, 10% FBS at 37°C with 5% CO₂ for 18 h and then treated with 0.05% DNBS for 2 h. The medium was removed and HaCaT cells were washed 3 times with 20 mM Tris-HCl (pH 7.4). Immediately, the cells were further incubated in fresh 90% RPMI 1640 containing 10% FBS for 6 h. The control HaCaT cells were not treated with 0.05% DNBS.

Protein extraction: Cell pellets were obtained at 3, 000 rpm for 5 min. The cells were then suspended and sonicated in ice-cold lysis buffer (7 Murea, 2 Mthiourea, 4% CHAPS, 30 mM Tris, a cocktail of protease inhibitor mixture). Supernatants were collected by centrifugation at 12,000 rpm for 15 min at 4°C. Proteins were extracted from three batches of cultures. Protein concentrations were determined using 2D Quant protein assay kit. The control and DNBS-treated samples were marked as C1, C2, C3, DNBS1, DNBS2 and DNBS3.

2D DIGE: Samples were labeled using minimal CyDye labeling according to our method (Liu et al., 2008; Sun et al., 2009). Equal amounts of protein extracts from each sample (total of 6) were pooled, and labeled with Cy2 as the internal standard, while Cy3 or Cy5 were used to label experimental or control samples (Table 1). Briefly, protein (50 μg) was labeled with 400 pmol of amine reactive cyanine dye. Labeling reactions were performed on ice in darkness for 30 min before quenched with 10 mM lysine. Unlabeled protein samples 150 μg were mixed with labeled protein (Table 1).The combined samples were then diluted with 2xrehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 2% IPG buffer, 20 mM DTT). 3-10 NL IPG strips (24cm) were subjected to IEF electrophoresis. Focusing was carried out: (I) 30 V, 12 h, step; (II) 300V, 3h, step; (III) 300-600V, 1350 Vh, gradient; (IV) 600-1000V, 2400Vh, gradient; (V)1000-8000V, 13500 Vh, gradient; ( ) 8000 V, 56000 Vh. Prior to the second dimension, the IPG strips were equilibrated twice with solutions containing 100 mM Tris- HCl (pH 8.0), 6 M urea, 30% glycerol, 2% SDS, and a trace amount of bromophenol blue. DTT (1%) and IAM (4.5%) were added in the solutions of the first and second equilibration steps, respectively. IPG strips were placed on top of 12.5% SDS-PAGE gels precast with low-fluorescence glass plates. SDS-PAGE was carried out under the following conditions: 0.2 W/gel for 1 h; 0.4 W/gel for 1 h; 1.8 W/gel for 15 h at 20°C.

Gel imaging and data analysis: Cy2, Cy3 and Cy5 labeled gels were individually imaged using excitation/ emission wavelengths of 488/520, 532/580 and 633/670 nm. Gel image was processed using DeCyder 6.5 (GE Healthcare, US). Spot detection was carried out using DIA module. BVA module was used for gel-to-gel matching, average abundance and statistics calculation. One Cy2-labeled internal standard spot map was served as the master gel image used to match different gel images (Alban et al., 2003; Quin et al., 2007; Richard et al., 2006). The proteins spots of interest with an average ratio more than 1.5 or less than -1.5,p<0.05, were selected and picked up for protein identification by mass spectrometry.

Deep purple staining: The gel with a silanized spacer low-fluorescence glass plate was subjected to Deep Purple staining (GE Healthcare) that enhances identification accuracy by mass spectrometry (Liu et al., 2008; Sun et al., 2009). The gel was fixed with 1000 mL fixing solution (7.5% HAC, 10% methanol) overnight, washed with 750 mL washing solution (2.94sodium bicarbonate, 31.8 g sodium carbonate, 750 mLH₂O), and stained with500 mL Deep Purple solutions for 1 h (Deep Purple:ddH₂O= 1:200) with vibration at RT in darkness. The gel was washed twice with500 mL of 7.5% HAC with vibration in darkness. Gel image was acquired using 488/610 nm.

Protein spots picking and in-gel digestion: Deep-Purple-stained gel image was matched to DIGE image, the spots of interest were robotically excised and digested according to our method (Liu et al., 2008; Sun et al., 2009). Briefly, the gel plugs were equilibrated with 25 mM ABC for 10 min and dehydrated with 10-min incubations with 100% ACN (twice). The dehydrated gel plugs were then incubated in 10 mM DTT at 56 °C for 45 min followed by induction 55 mM IAM for 45 min. Repeat twice the first wash and dehydrate steps. Protein was digested with porcine modified trypsin (Promega) in 25 mM ABC for 16 h at 37 °C. Tryptic peptides were extracted from gel in two cycles of 50% ACN, 1% FA.

Protein identification by mass spectrometry: An HPLC chromatography (Surveyor) and an LTQ mass spectrometer combined platform was used for HPLC-nESI-MS/MS. A nanoscale C18 analytical column (150 ×
0.075mm, Dionex) was set for HPLC. Mobile phase A was 5% ACN with 0.1% FA, mobile phase B was 95% ACN with 0.1% FA. Peptides were eluted at a flow rate of 260 nL/min with a linear gradient of 5–90% B over 50 min. Mass spectra were collected using data-dependent acquisition of one MS full scan (400 to 1700 m/z) followed by MS/MS scans of the five most abundant ions. MS/MS spectra were interpreted using Turbo Sequest Bioworks. The peptide cross-correlation scores (Xcorr) of 1.9, 2.5, and 3.75 were set for charge states 1, 2, and 3. Protein probability was set <10^−3. Cut-off threshold of the number of matched peptides per protein was set two, additional peptides were included to maximize peptide coverage of identified protein (Liu et al., 2008; Sun et al., 2009).

MALDI MS protein identification was performed based on PMT (peptide mass fingerprinting) assay using a ProTOF 2000 mass spectrometer according to our method (Liu et al., 2006; Sun et al., 2006). In-gel tryptic peptides of spots were desalted and concentrated through a ZipTip C18. Peptides eluted from tip were mixed with α-cyano-4-hydroxycinnamic acid (10 mg/ml) in 50% acetonitrile with 0.1% TFA at volume ratio of 1:1. Mass spectrometer was operated in a 16 kV positive mode with am/z range of 700–4000.

SDS-PAGE and Western blotting: Harvested HaCaT and DNBS-treated HaCaT cells were washed with cold PBS 3 times, and were sonicated in ice-cold RIPA buffer (Santa Cruz, US) for 20 min. The supernatants were collected by centrifugation at 12,000 rpm for 15 min at 4°C. The protein concentrations of samples were determined by Bradford Assay.

Same amount of proteins from each sample was fractionated by 12% SDS-PAGE and transferred onto nitrocellulose membranes. β-actin was used as the internal standard. Membranes were washed three times for 10 min with PBS-T (20 mM, pH 7.4, 100 mM NaCl, 0.5% Tween-20) and blocked with nonfat milk powder 5% milk PBS-T (0.5% Tween-20) for 1 h at RT. Being washed extensively with PBS-T, the membranes were then incubated with the horseradish peroxidase-coupled secondary antibodies and were developed by ECL according to the kit instructions (GE HealthCare, US) using a ChemiDoc™ MP image system (Bio-Rad, US). And the relative expression levels of identified proteins were quantified by the densitometry of visualized bands versus that of the internal standard using Image Lab software (Bio-Rad, US). The antibodies used were as follows: rabbit anti-human CALM3 polyclonal antibody (1:350, ProteinTech), rabbit anti-p23 monoclonal antibody (1:1200, Abcam), mouse anti-IL-18 monoclonal antibody (1:400, Abcam), rabbit anti-TCEB2 polyclonal antibody (1:2000, ProteinTech), goat anti-MYL6 polyclonal antibody (1:200, Santa Cruz), rabbit anti-BANF1 monoclonal antibody (1:2000, Abcam) and rabbit anti-RPL23 polyclonal antibody (1:600, ProteinTech).

**RESULTS**

2D DIGE gel patterns were consistent and reproducible. Over 1200 protein spots were averagely resolved in each of the 3 gels, and over 800 protein spots were matched in all 3 gels. A comprehensive gel views of DIGE were shown in Fig. 1, with gel images of Cy2-labelled internal standard (Fig. 1A), Cy3-labelleduntreated HaCaT (Fig. 1B), Cy5-labelledDNBS-treated HaCaT (Fig. 1C) and overlaid gel image (Fig. 1D). Deep Purple-stained gel image was given in Fig. 1E. Deep Purple was used for gel post-staining as proteins were statistically more accurately identified by mass spectrometry with higher quality (Liu et al., 2008; Sun et al., 2009). DIA analysis of protein expression in DIGE gels produced 3-D representations of protein spots proportional in volume to the emission florescence intensity. Grounded on DeCyder analysis, the ratios of normalized spot intensities of DNBS-treated HaCaT untreated HaCaT cells were calculated. Nine spots showing more than 1.5- or less than -1.5-fold difference (p<0.05) were picked (numbered in DIGE image, Fig.1F). The volumes of 7 spots were increased and 2 spots were decreased for DNBS-treated HaCaT cells comparing to control cells (Table 2).

Eight unique proteins were identified by searching MS/MS data against *Homo sapiens* database derived from NCBI database through TurboSequest Bioworks (Table 2). Among identified proteins, the 6 most up-regulated proteins were chromosome X open reading frame 26 (Cxor26), calmodulin (CALM3), human co-chaperone P23 (PTGES3), smooth muscle and non-muscle myosin alkali light chain (MYL6) and breakpoint cluster region protein 1 (BANF1) and IL-18, the 2most down-regulated proteins were elongin B isoform alpha (TCEB2) and ribosomal protein
L23(RPL23).

Fig. 2 shows a representative identification result for spot 5 of CALM3. Seven peptides, LTDEEVDEM*IR, VFDKDNGYISAELR, M*KDTSEEEIREAFR, DT DSEEIREAFR, MKDTSEEEIR, LTDEEVDEMIR, E AFSLFDKDGDTITIK and EADIDDGQVYTEEFV QM*M'TAK were derived from MS data (*methionine oxidation, Fig. 2A). Fig. 2B is the representative MS/MS spectrum of VFDKDNGYISAELR. The peaks at mass-to-charge (m/z), 288.26, 417.16, 488.48, 599.39, 649.39, 759.49, 922.44, 979.50, 1094.52, 1151.48, 1266.51 and 1339.42 were the y series of fragment ions of y2, y3, y4, y5, y6, y7, y8, y9, y10, y11, y12 and y13. The peaks at mass-to-charge (m/z), 605.32, 662.25, 777.30, 833.11, 997.37, 1110.37, 1468.49 and 1581.66 were the b series of fragment ions of b5, b6, b7, b8, b9, b10, b14 and b15. The peaks 587.34 and 1249.71 were confirmed as dehydration fragment ions of b5 and b12, and 1076.62 and 1450.46 were confirmed as deamination fragment ions of y10 and b14 (Fig. 2B). Spots 3 and 4 were identified as PTGES3 (Table 2). Same protein was identified from different spots might be caused by protein isoforms and/or protein post-translational modifications.

Figure 1. Representative images of 2D DIGE gels.
(A) gel image of Cy2-labelled internal standard; (B) gel image of Cy3-labelleduntreated HaCaT cell; (C) gel image of Cy5-labelled DNBS treated HaCaT cell; (D) overlayed gel image of Cy2-, Cy3- and Cy5-labelled protein extracts of HaCaT cells; E: gel image stained with Deep Purple; (F) differentially expressed protein spots.

The identified proteins were associated with HaCaT DNBS damage, which also implicates they might be potential targets for skin cell damage and skin diseases caused by chemical carcinogens. To validate the protein identification results by proteomic approach, the densitometric quantification results for expression levels detected by Western blotting (WB) for PTGES3, CALM3, TCEB2, IL18, BANF1, MYL6 and RPL23 are shown in Fig.3. WB assays indicated that the levels of PTGES3, CALM3, TCEB2, IL18, BANF1, MYL6 and RPL23 were about 2.0-, 1.8-, 0.59-, 1.6-, 2.7-, 2.1- and 0.53-folds in DNBS-stimulated HaCaT cells relative to HaCaT (Fig.
The WB validation results were consistent with proteomic results of the expression fold changes of 1.6 and 1.5 (up-regulation, spots 3 and 4) for PTGES3, 1.8 of CALM3 (up-regulation, spot 5), 2.0 of TCEB2 (down-regulation, spot 6), 1.5 of IL18 (up-regulation, spot 2), 1.8 of BANF1 (up-regulation, spot 8) 1.5 of MYL6 (up-regulation, spot 7) and 1.5 of RPL23 (down-regulation, spot 9), as shown in Table 2. The changes measured by WB demonstrate similar trends as reported by 2-DE DIGE. Meanwhile, the mRNA levels of 8 identified corresponding proteins show same expression trends as their protein levels. qRT-PCR results showed the mRNA expression levels of CXorf26, IL18, PTGES3, CALM3, MYL6 and BANF1 increased to be ~2.8-, 3.3-, 4.6-, 3.9-, 2.2- and 5.8-fold in DNBS-treated HaCaT cells compared to control group HaCaT cells. While, the mRNA levels of TCEB2 and RPL23 in DNBS-treated HaCaT cells were only ~32% and 42% in HaCaT cells due to the stimulation of DNBS. qRT-PCR results are also consistent with DIGE and Western blotting results.

Figure 2. MS identification result for gel spot 1128.
(A) Overall identification result for 1128. (B) MS/MS spectrum of identified peptide VFDKDNGYISAELR.
Table 1. Compositions and gel assignments of the six samples for 2D DIGE.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Cy2 (Ins)</th>
<th>Cy3 labeling</th>
<th>Cy5 labeling</th>
<th>Unlabeled protein</th>
<th>a</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50 µg</td>
<td>50 µg C1</td>
<td>50 µg DNBS1</td>
<td>150 µg</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>50 µg</td>
<td>50 µg DNBS2</td>
<td>50 µg C2</td>
<td>150 µg</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>50 µg</td>
<td>50 µg C3</td>
<td>50 µg DNBS3</td>
<td>150 µg</td>
<td></td>
</tr>
</tbody>
</table>

*8.33 (50/6, 6 means the 6 samples) µg of each samples from C1, C2, C3, DNBS1, DNBS2 andDNBS3 were pooled together; 25 µg of each samples from C1, C2, C3, DNBS1, DNBS2 andDNBS3.

Table 2. Up- and down-regulated Proteins in DNBS-Treated group Compared with Control Group

<table>
<thead>
<tr>
<th>Spot No.</th>
<th>Protein Description</th>
<th>Gene Symbol</th>
<th>Accession No.</th>
<th>Peptides Matched</th>
<th>Sequence Coverage (%)</th>
<th>Deregulation (P&lt;0.05)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Chromosome X open reading frame 26</td>
<td>CXorf26</td>
<td>30354299</td>
<td>13/32</td>
<td>51</td>
<td>1.5↑</td>
</tr>
<tr>
<td>2</td>
<td>Interferon-gamma inducing factor precursor</td>
<td>IL18</td>
<td>1899242</td>
<td>13/47</td>
<td>52</td>
<td>1.5↑</td>
</tr>
<tr>
<td>3</td>
<td>Human co-Chaperone P23</td>
<td>PTGES3</td>
<td>9257073</td>
<td>4/62</td>
<td>38</td>
<td>1.6↑</td>
</tr>
<tr>
<td>4</td>
<td>Human Co-Chaperone P23</td>
<td>PTGES3</td>
<td>9257073</td>
<td>7/48</td>
<td>64</td>
<td>1.5↑</td>
</tr>
<tr>
<td>5</td>
<td>Calmodulin</td>
<td>CALM3</td>
<td>825635</td>
<td>8/27</td>
<td>54</td>
<td>1.8↑</td>
</tr>
<tr>
<td>6</td>
<td>Transcription elongation factor B polypeptide 2 isoform</td>
<td>TCEB2</td>
<td>6005890</td>
<td>5/14</td>
<td>35</td>
<td>-2.0↓</td>
</tr>
<tr>
<td>7</td>
<td>Myosin light polypeptide 6</td>
<td>MYL6</td>
<td>16924329</td>
<td>10/30</td>
<td>53</td>
<td>1.5↑</td>
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<tr>
<td>8</td>
<td>Breakpoint cluster region protein 1</td>
<td>BANF1</td>
<td>3002951</td>
<td>7/17</td>
<td>37</td>
<td>1.8↑</td>
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<tr>
<td>9</td>
<td>Ribosomal protein L23</td>
<td>RPL23</td>
<td>38571606</td>
<td>2/4</td>
<td>24</td>
<td>-1.5↓</td>
</tr>
</tbody>
</table>

* Protein fold changes between DNBS-treated HaCaT and control HaCaT cells. 1.5↑ and -1.5↓ represent 50% increase and 50% decrease in protein expressions for treated sample than the control; *represents the gene identification number. Note: Spots 3 and 4 were identified by MALDI MS; Spots 1,2,5,6,7,8 and 9 were identified by HPLC-EST-MS/MS.

Table 3. Designed primers of target genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
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<tr>
<td>CXorf26</td>
<td>F:5'-GAACTCAAGTCAGATCAGCAGC-3'</td>
</tr>
<tr>
<td></td>
<td>R:5'-AGAACAATCTAGTCGAGGAGC-3'</td>
</tr>
<tr>
<td>IL-18</td>
<td>F:5'-GACTGTAGAGATAGGATGCACC-3'</td>
</tr>
<tr>
<td></td>
<td>R:5'-GAGATAGTTACAGCCTACATCCT-3'</td>
</tr>
<tr>
<td>PTGES3</td>
<td>F:5'-AAACTTACATTCAGTTGTCCTCGG-3'</td>
</tr>
</tbody>
</table>
DISCUSSION

DIGE technique is one of the most reliable quantification proteomic methods (Liu et al., 2008; Sun et al., 2009). Two different samples and a pooled standard are separated in a single DIGE gel that minimizes the reproducibility problem. The quantification of protein expression profile can be rapidly and accurately achieved based on the emission fluorescence intensities of Cy2, Cy3 and Cy5. The inclusion of pooled standard sample on each gel facilitates precise gel-to-gel matching, as same matched complete spot map is present on each gel (Liu et al., 2008; Sun et al., 2009).

Among 8 protein candidates, CALM3 was reported to be associated with skin burns and wound healing, keratinocytes proliferation and migration. Rare study has been addressed on the associations of remaining proteins with skin cell damages and skin diseases. CALM3 was involved in terminal keratinocyte differentiation (Rosenthal et al., 2000). It inhibits the proliferation of dividing human keratinocytes. CALM3 mRNA level decreased by 30% and 50% in primary human endothelial cells treated with UV-irradiation for 1 h and 3 h (Watson et al., 2000). It was down-regulated in HeLa cells in response to UV-irradiation, while went back to normal after 24 h of UV-irradiation treatment. CALM3 level change correlated to DNA replication activity and synchronizes DNA synthesis (Rosenthal et al., 2000; Watson et al., 2000; Watson et al., 2000). Interestingly, our results showed it was up-regulated in response to DNBS stimulation. Further study is required for its action mechanism.

The rest identified proteins were for the first time reported to be associated with skin cell damage. Myosin is a motor protein playing a key role in cell migration and posterior cell retraction. MYL was abnormally expressed in UVC-irradiated HeLa cells (Decker et al., 2003). Myosin II phosphorylation was important for wounded keratinocytes cell migration and epidermis wound healing (Betapudi et al., 2010; Iocono et al., 2003). Myosin-10 could be activated by calmodulin-like protein to function in skin differentiation and wound re-epithelization of human keratinocytes (Bennett et al., 2009). DNBS stimulation affected MYL6 expression in HaCaT cells. MYL6 insp07 was up-regulated to 1.5-fold of that in control HaCaT cells (Table 2).

UV-induced immune suppression is a risk factor for skin cancer development (Hur et al., 2010; Katiyar 2007; Yanti and Hwang, 2010). The normal level of cytokines interleukin 8 (IL-8) was critical for skin wound healing. IL-8 down-regulation in wounded skin or unhealed skin resulted in decreased keratinocyte replication. However, the elevated IL-8 levels contributed to retarded wound healing (Iocono et al., 2000). IL-12 removes or repairs UV-induced DNA damage in skin (Katiyar 2007). IL-18 plays important roles in cancers, in autoimmune and infectious diseases (Gracie et al., 2003). IL-18 was up-regulated in HaCaT cells following DNBS stimulation, suggesting its association with skin cell damage or even skin diseases.

Heat shock proteins (HSPs) are involved in protein misfolding prevention and repair, and cell injuries. HSP27, HSP70 and HSP90 were up-regulated in UVB-irradiated HeLa cells counteracting the damage through interactions with DNA repair enzymes (Mendez et al., 2000; Mori-Iwamoto et al., 2007; Yang et al., 2009). As an important modulator of HSP90 activity, PTGES3 is a prominent target in cell apoptosis inhibiting the aggregation of denatured proteins (Gausdal et al., 2009). Our proteomic data indicated PTGES3 was up-regulated to 1.6-fold, which was the direct response from HaCaT against DNBS. It might be a potential marker for skin cell damage by chemical carcinogens.

There are no study reported on the associations of TCEB2, CXorf26, BANF1 and RPL23, as identified differentially expressed in HaCaT against DNBS stimulation (Table 2), with skin cell damages or skin diseases. These protein candidates might be potential indicators for skin cell damage and new targets in chemical carcinogens-induced skin diseases.

Conclusions: Chemical carcinogens are playing important roles in the development of human skin
diseases. CXorf26, IL18, PTGES3, CALM3, TCEB2, MYL6, RPP2, BANF1 and RPL23 were most differentially expressed in HaCaT proteome following DNBS stimulation. CALM3 was associated with skin burn wound healing, keratinocytes proliferation and migration. PTGES3 up-regulation in HaCaT cells might help cell to remove and repair misfolded proteins caused by DNBS stimulation. The abnormal expression of IL-18 implicates an immune response in HaCaT against possible inflammation induced by DNBS.

Most of these identified protein candidates in HaCaT cells were for the first time revealed responsible for DNBS stimulation damage. The precise roles of them in skin cell against chemical carcinogens need investigating. They are potential effective protection indicators for skin diseases induced by chemical carcinogens.

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