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ORIGINAL PAPER

Gene expression profiling of hair-dying agent, para-phenylenediamine, in human keratinocytes (HaCaT) cells

Young-Jin So[1], Chan-Young Shin[2], Mee Song[2], Young-Ah Rha[3] & Jae-Chun Ryu[2]

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Abstract Para-phenylenediamine (PPD) is widely used in a variety of products, including hair dyes. Sensitization to PPD is a well-known cause of allergic contact dermatitis. However, the molecular mechanism of PPD-induced cellular toxicity remains unclear. We performed a genome-wide analysis of transcriptional responses of human HaCaT keratinocytes to an IC20 dose of PPD (60 μM). PPD downregulated the expression of 650 genes and upregulated the expression of 854 genes in the keratinocytes. PPD-responsive genes were involved in inflammatory responses, responses to oxidative stress, Toll-like receptor signaling, and metabolism of xenobiotics by cytochrome P450. Our results indicate that changes in gene expression are associated with PPD-induced skin toxicity, and the genes identified may be promising biomarkers of PPD-induced skin toxicity. Possible roles of the responsive genes and related pathways in PPD-induced cellular responses are discussed. This study helps to elucidate cellular changes in transcriptional regulation in response to PPD exposure.

Keywords Para-phenylenediamine (PPD; 1,4-diaminobenzene; CAS 106-50-3), Keratinocytes, Microarray, Gene ontology (GO)

[1]Department of Dermatological Health Management, Eulji University, Seongnam 461-713, Korea
[2]Cellular and Molecular Toxicology Laboratory, Korea Institute of Science & Technology P.O. Box 131, Cheongryang, Seoul 130-650, Korea
[3]Department of Food Technology and Services, Eulji University, Seongnam 461-713, Korea
Correspondence and requests for materials should be addressed to J. C. Ryu (✉ryujc@kist.re.kr)

Para-phenylenediamine (PPD) is an aromatic amine widely used in the pharmaceutical, chemical, rubber, dye, textile, and photographic industries[1,2]. PPD is present in about 70% of all hair dyes worldwide[3]. PPD polymerizes within the hair shaft in the presence of a coupler, causing a change in hair color. Under oxidative hair-dyeing conditions, the production of nonreactive, high-molecular-weight, polymerized hair-coloring molecules is chemically favored owing to an excess of available couplers[4-6]. After the formation of the coloring molecules is complete, typically less than 1% of the agent remains[7].

PPD is well known as a skin irritant and sensitizer, and allergic contact dermatitis may occur after exposure to PPD in hair dye[8-13]. Exposure occurs predominantly via the skin, where PPD is absorbed by epidermal cells. Local contact with PPD can cause skin irritation, contact dermatitis, chemosis, lacrimation, exophthalmos, and even permanent blindness[14]. Sensitization to PPD occurred in 2.5% to 4% of individuals with eczema, and a sensitization rate of 10% was reported in occupationally exposed individuals with skin diseases[15].

PPD belongs to a family of chemicals with a wide spectrum of toxicological properties. These chemicals include human and animal carcinogens, as well as non-carcinogens[16-20], and PPD itself shows little mutagenic effect[21]. PPD can be oxidized to benzoquinone diamine, which in turn forms the trinuclear dye *N,N′*-bis(4-aminophenyl)-2,5-diamino-1,4-quinone-diimine, or Bandrowski's base (BB)[21]. BB has been reported to be the immunogen in PPD allergy[15]. PPD can also be acetylated to mono-acetyl-PPD and di-acetyl-PPD by *N*-acetyltransferases in human skin and keratinocytes[7]. The *N*-acetylated derivatives of PPD may be susceptibility factors for the development of allergy

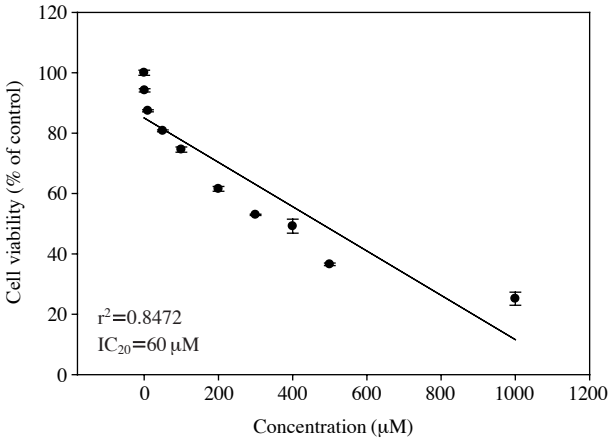

Figure 1. Cell viability measured by MTT assay. HaCaT cells were exposed to different concentrations of PPD for 48 h. After exposure, cell viability for each treatment was determined based on spectrometry of formazan formation, and represented the viability percentage relative to control (DMSO) exposure.

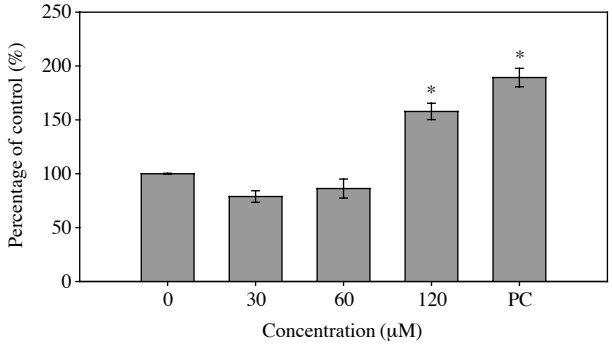

Figure 2. Changes in ROS formation in HaCaT cells upon exposure to 1/2 of IC_{20}, IC_{20} and 2 of IC_{20} doses PPD. Fluorescence intensity of DCFH-DA was measured at wavelength of 485/530 (Ex/Em) and normalized to total cell viability detected by MTT assay. Values that are significantly different from sample to sample are indicated by $*P$-value < 0.05 compared to control and presented as mean ± SD. Values are the means of three determinations on each of the three replicate exposures and expressed as percentage of control. Positive control (PC) is 2 mM H_2O_2.

to PPD[21]. Previous studies of the biological mechanisms of PPD allergy have suggested that decreased *N*-acetyltransferase-mediated *N*-acetylation of PPD and induction of cyclooxygenase expression and activity by PPD, likely via oxidative processes, are involved in PPD-related skin inflammation and allergy[7,21]. Despite a long-standing recognition of allergy to PPD, the action mechanisms of PPD *in vitro* and *in vivo* have remained elusive. PPD-induced changes in gene expression related to its mechanism of action and prediction of toxicity have rarely been reported.

In the present study, microarrays were used to study overall changes in transcription profiles of human HaCaT keratinocytes in response to PPD. The aims of this study were to identify differentially expressed genes (DEGs) in human keratinocytes and potential gene-based biomarkers for PPD allergy, and to better understand the mechanism of action of PPD in the main target organ and cells involved in allergic dermatitis, namely the skin and keratinocytes.

Cytotoxicity of PPD in HaCaT cells

To determine suitable concentrations of PPD for use in the experiments, the relative survival of HaCaT cells following exposure to PPD at a range of concentrations was determined by MTT assay. Relative survival was determined as the optical density of treated cells as a percentage of the optical density of solvent (DMSO)-treated cells. Dose-dependent HaCaT cell viability curves were obtained after exposure to PPD for 48 h (Figure 1). The PPD concentration producing 20% inhibition of cell viability (IC_{20}) was 60 μM.

Induction of ROS generation after exposure to PPD

Previous studies have reported that the induction of reactive oxygen species (ROS) generation may be related to PPD skin toxicity[22]. We determined whether PPD induces ROS generation in a dose-dependent manner. PPD at an IC_{20} dose (60 μM) did not induce ROS production in HaCaT cells (Figure 2). However, treatment of HaCaT cells with 120 μM PPD did induce ROS production (Figure 2).

Gene expression profiles of HaCaT cells after exposure to PPD

In this study, we used an IC_{20} dose of PPD. At this dose, PPD can induce changes in gene transcription without inducing ROS generation, allowing an analysis of altered gene expression due only to PPD in skin toxicity. To identify global changes in gene expression related to PPD exposure, microarray analysis was performed using Agilent human 8 × 60 K whole genome microarrays. A difference in gene expression between treated and control cells was defined as a fold change of ≥ 1.5 at $P < 0.05$. A total of 1,504 differentially expressed genes (DEGs) were identified in HaCaT cells treated with 60 μM PPD for 48 h, compared with control cell gene expression. Of these genes, 854 were upregulated and 650 were downregulated in response to treatment with an IC_{20} dose of PPD. These results indicate that exposure to a low dose of PPD can induce various changes in gene expression.

Biological function of DEGs in PPD-treated HaCaT cells

To analyze the possible mechanism of PPD-induced skin toxicity, DAVID (http://david.abcc.ncifcrf.gov) was used to search gene ontology (GO) databases and the literature for the DEGs induced by PPD. To select overrepresented GO biological processes, the DEGs identified in DAVID were classified using Fisher's exact test with a cut-off of $P<0.01$. Significantly enriched GO biological process categories for genes upregulated by PPD included the following: M phase, chromosome segregation, spindle organization, phosphoinositide-mediated signaling, lipid biosynthetic process, inflammatory response, response to oxidative stress, cell proliferation, membrane lipid metabolic process, cytoskeleton organization, DNA repair, angiogenesis, and unsaturated fatty acid metabolic process (Table 1). Significantly enriched GO categories for genes downregulated by PPD were: regulation of cell proliferation, hair follicle morphogenesis, cell morphogenesis involved in differentiation, gland development, collagen metabolic process, cell motility, regulation of cell migration, and epidermis development (Table 1).

The genes that were upregulated and downregulated by PPD were also classified according to the PATHWAY database of the Kyoto Encyclopedia of Genes and Genomes (KEGG), to identify molecular mechanisms related to PPD exposure. Upregulated genes were positively associated the following pathways: cell cycle, p53 signaling pathway, arachidonic acid metabolism, steroid hormone biosynthesis, oocyte

Table 1. Significantly enriched GO categories for PPD-induced genes.

UP			Down		
GO term	Number	*P*-value	GO term	Number	*P*-value
M phase	52	0.00000	regulation of cell proliferation	36	0.00006
chromosome segregation	19	0.00000	hair follicle morphogenesis	5	0.00069
spindle organization	9	0.00018	cell morphogenesis involved in differentiation	14	0.00299
phosphoinositide-mediated signaling	12	0.00031	gland development	10	0.00300
lipid biosynthetic process	25	0.00065	collagen metabolic process	5	0.00311
inflammatory response	25	0.00070	cell motility	16	0.00338
response to oxidative stress	16	0.00086	regulation of cell migration	11	0.00426
cell proliferation	29	0.00225	epidermis development	11	0.00765
membrane lipid metabolic process	10	0.00247			
cytoskeleton organization	28	0.00440			
DNA repair	20	0.00715			
angiogenesis	13	0.00722			
unsaturated fatty acid metabolic process	7	0.00964			

Shown are Gene Ontology (GO) categories with $P \leq 0.01$. Numbers in parenthese are numbers of genes in the GO category.

Table 2. KEGG annotations for PPD-induced genes.

UP			Down		
GO term	Number	*P*-value	GO term	Number	*P*-value
Cell cycle	19	0.00000	Basal cell carcinoma	5	0.03309
p53 signaling pathway	11	0.00024	Hedgehog signaling pathway	5	0.03505
Arachidonic acid metabolism	8	0.00530	Melanogenesis	6	0.06839
Steroid hormone biosynthesis	7	0.00786	Intestinal immune network for IgA production	4	0.09439
Oocyte meiosis	11	0.00942			
Glutathione metabolism	7	0.01175			
Homologous recombination	5	0.02122			
Glycerolipid metabolism	6	0.02829			
Toll-like receptor signaling pathway	9	0.04006			
Retinol metabolism	6	0.05563			
Cytosolic DNA-sensing pathway	6	0.05936			
Tryptophan metabolism	5	0.06647			
Pathways in cancer	19	0.07979			
Metabolism of xenobiotics by cytochrome P450	6	0.08012			
Lysosome	9	0.08094			

Shown are KEGG pathway categories with $P \leq 0.1$. Numbers in parenthese are numbers of genes in the KEGG pathway category.

Table 3. Key functions of genes in PPD-treated HaCaT cells.

GeneBank Accession No.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)
Inflammatory response			
NM_015364	*LY96*	lymphocyte antigen 96	3.666
NM_005621	*S100A12*	S100 calcium binding protein A12	3.637
NM_021187	*CYP4F11*	cytochrome P450, family 4, subfamily F, polypeptide 11	3.608
NM_002964	*S100A8*	S100 calcium binding protein A8	3.394
NM_005252	*FOS*	FBJ murine osteosarcoma viral oncogene homolog	3.188
NM_006072	*CCL26*	chemokine (C-C motif) ligand 26	3.079
NM_138554	*TLR4*	toll-like receptor 4	2.860
NM_000600	*IL6*	interleukin 6 (interferon, beta 2)	2.506
NM_002562	*P2RX7*	purinergic receptor P2X, ligand-gated ion channel, 7	2.195
NM_002985	*CCL5*	chemokine (C-C motif) ligand 5	2.166
NM_001557	*CXCR2*	chemokine (C-X-C motif) receptor 2	2.049
NM_002965	*S100A9*	S100 calcium binding protein A9	1.952
NM_001042483	*NUPR1*	nuclear protein, transcriptional regulator, 1	1.936
NM_001734	*C1S*	complement component 1, s subcomponent	1.875
NM_201442	*C1S*	complement component 1, s subcomponent	1.868
NM_007283	*MGLL*	monoglyceride lipase	1.813
NM_002983	*CCL3*	chemokine (C-C motif) ligand 3	1.803
NM_013314	*BLNK*	B-cell linker	1.795
NM_000952	*PTAFR*	platelet-activating factor receptor	1.793
NM_000576	*IL1B*	interleukin 1, beta	1.732
NM_002133	*HMOX1*	heme oxygenase (decycling) 1	1.633
NM_197954	*CLEC7A*	C-type lectin domain family 7, member A	1.539
NM_001014975	*CFH*	complement factor H	1.516
NM_173843	*IL1RN*	interleukin 1 receptor antagonist	1.504
NM_000575	*IL1A*	interleukin 1, alpha	1.501
NM_198336	*INSIG1*	Insulin induced gene 1	0.26
Response to oxidative stress			
NM_002963	*S100A7*	S100 calcium binding protein A7	4.359
NM_005252	*FOS*	FBJ murine osteosarcoma viral oncogene homolog	3.188
NM_138554	*TLR4*	toll-like receptor 4, transcript variant 1	2.860
NM_002985	*CCL5*	chemokine (C-C motif) ligand 5	2.166
NM_000962	*PTGS1*	prostaglandin-endoperoxide synthase 1 (prostaglandin G/H synthase and cyclooxygenase), transcript variant 1	2.117
NM_000903	*NQO1*	NAD (P)H dehydrogenase, quinone 1, transcript variant 1	1.964
NM_000402	*G6PD*	glucose-6-phosphate dehydrogenase , transcript variant 1	1.858
NM_001498	*GCLC*	glutamate-cysteine ligase, catalytic subunit, transcript variant 1	1.819
NM_000963	*PTGS2*	prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)	1.797
NM_024420	*PLA2G4A*	phospholipase A2, group IVA (cytosolic, calcium-dependent)	1.731
NM_002133	*HMOX1*	heme oxygenase (decycling) 1	1.633
NM_002228	*JUN*	jun proto-oncogene	1.575
NM_080725	*SRXN1*	sulfiredoxin 1	1.561
NM_005438	*FOSL1*	FOS-like antigen 1	1.560
NM_001008397	*GPX8*	glutathione peroxidase 8 (putative)	1.538
NM_139266	*STAT1*	signal transducer and activator of transcription 1, 91 kDa, transcript variant beta	1.537
Toll-like receptor signaling pathway			
NM_015364	*LY96*	lymphocyte antigen 96	3.666
NM_005252	*FOS*	FBJ murine osteosarcoma viral oncogene homolog	3.188
NM_138554	*TLR4*	toll-like receptor 4	2.860
NM_000600	*IL6*	interleukin 6 (interferon, beta 2)	2.506
NM_002985	*CCL5*	chemokine (C-C motif) ligand 5	2.166
NM_002983	*CCL3*	chemokine (C-C motif) ligand 3	1.803
NM_000576	*IL1B*	interleukin 1, beta	1.732
NM_002228	*JUN*	jun proto-oncogene	1.575
NM_139266	*STAT1*	signal transducer and activator of transcription 1, 91 kDa	1.537

Table 3. Continued.

GeneBank Accession No.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)
Metabolism of xenobiotics by cytochrome P450			
NM_001818	*AKR1C4*	aldo-keto reductase family 1, member C4 (chlordecone reductase; 3-alpha hydroxysteroid dehydrogenase, type I; dihydrodiol dehydrogenase 4)	4.278
NM_001353	*AKR1C1*	aldo-keto reductase family 1, member C1 (dihydrodiol dehydrogenase 1; 20-alpha (3-alpha)-hydroxysteroid dehydrogenase)	4.137
NM_003739	*AKR1C3*	aldo-keto reductase family 1, member C3 (3-alpha hydroxysteroid dehydrogenase, type II)	3.761
NM_000849	*GSTM3*	glutathione S-transferase mu 3 (brain)	3.204
XR_111839	*FAM125A*	family with sequence similarity 125, member A	1.763
NM_000499	*CYP1A1*	cytochrome P450, family 1, subfamily A, polypeptide 1	1.760
NM_019076	*UGT1A8*	UDP glucuronosyltransferase 1 family, polypeptide A8	1.630
NM_001072	*UGT1A6*	UDP glucuronosyltransferase 1 family, polypeptide A6	1.559

meiosis, homologous recombination, glycerolipid metabolism, Toll-like receptor signaling pathway, retinol metabolism, cytosolic DNA-sensing pathway, tryptophan metabolism, pathways in cancer, metabolism of xenobiotics by cytochrome P450, and lysosome (Table 2). The pathways positively associated with downregulated genes included: basal cell carcinoma, hedgehog signaling pathway, melanogenesis, and intestinal immune network for IgA production (Table 2).

Among the biological functions identified by the GO biological process and KEGG pathway analyses, several key functions related to PPD-responsive genes were identified, including inflammatory response, response to oxidative stress, and hair follicle morphogenesis as GO biological processes, and Toll-like receptor signaling pathway and metabolism of xenobiotics by cytochrome P450 as KEGG pathways. Table 3 shows the genes involved in biological processes related to the PPD response in HaCaT cells.

Discussion

PPD and related chemicals are common contact sensitizers and frequently cause allergic contact dermatitis. Nevertheless, the chemical mechanism involved has remained elusive. Studies regarding the effects of PPD on gene expression have mainly focused on contact dermatitis.

Characterizing PPD-induced changes in gene expression patterns and understanding the mechanisms of PPD-induced toxicity may provide markers for the early identification of relevant toxicological findings in compound screens and may aid in the development of therapeutics to reduce skin toxicity. The aim of this study was to identify genes involved in the biological response to PPD and to elucidate the response mechanisms in mammalian cells to PPD, using toxicogenomic technology[23]. Statistical analyses of microarray results revealed a number of DEGs associated with PPD-mediated skin toxicity.

PPD has been reported to influence the expression of the *IFNG, IL10, IL4, NAT1, PTGS1, PTGS2*, and *TNF* genes[24-26]. *IFNG* (interferon-γ) and *IL4* (interleukin-4) were correlated with allergic contact dermatitis caused by PPD, and the cytokine tumor necrosis factor (TNF)-α plays a key role in contact sensitization[10,28]. According to the Comparative Toxicogenomics Database, PPD increases the expression of the following genes: *ABCC3, ANXA1, ATF3, CD86, CDKN1A, DUSP1, FTH1, GABARAPL3, GCLM, HIF1A, HMOX1, HTATIP2, IFNG, IL10, IL4, ME1, MT1H, MT2A, NAT1, NFE2L2, NINJ1, NQO1, PGD, PIR, PLIN2, PMAIP1, PPARG, PTGS1, PTGS2, RIT1, S100P, SI,* and *SPTA1*. In addition, PPD reduces the expression of the chemokine receptor genes *CCR6* and *CXCR4*, and disrupts *TNF* polymorphisms.

In the present study, we used human 8 × 60 K whole genome microarrays to analyze the gene expression profile of HaCaT cells treated with PPD, and identified 854 upregulated genes and 650 downregulated genes following PPD exposure. Database analyses suggested several biological processes and pathways associated with these DEGs. The focus of this discussion will be on gene expression changes associated with the inflammatory response, the response to oxidative stress, glutathione metabolism, the Toll-like receptor signaling pathway, and metabolism of xenobiotics by cytochrome P450 (Table 3).

Toll-like receptors (TLRs) are important pattern-recognition receptors involved in the defense against chemical exposure[27,28]. The activation of TLRs leads

to the production of cytokines, chemokines, and adhesion molecules that participate in innate and adaptive immune responses[27]. TLRs play key roles in the pathophysiology of various inflammatory skin diseases[28]. We found that PPD increased the expression of *TLR4* in human keratinocytes. The activation of TLR4 on keratinocytes during the immune response induces the production of TNF-α and IL-8[27]. Keratinocytes are responsive to cytokines, including interferons, TNF-α, IL-17, and the IL-20 subfamily of cytokines. These stimuli induce the expression of pro-inflammatory cytokines (e.g., IL-1, IL-6, and TNF-α) as well as chemokines such as CXCL8 (IL-8), CXCL10, and CCL29, which are small heparin-binding proteins with chemotactic activity[29]. According to our gene expression data, PPD induced the expression of chemokine (*CCL26, CCL5*, and *CCL3*) and cytokine genes (*IL6, IL1B,* and *IL1A*). These chemokines and cytokines may induce an inflammatory response in keratinocytes exposed to PPD.

Previous studies have reported that PPD may cause lipoperoxidative damage and modify the intracellular antioxidant pool, which may be important in the pre-immunological phase of contact sensitization[3]. Xenobiotic-metabolizing enzymes such as cytochrome P450 (CYP) enzymes have been linked to skin toxicity and immune response-like allergic contact dermatitis[32]. CYP1 isoenzymes, including CYP1A1, CYP1A2, and CYP1B1, are critical for the metabolic activation of many xenobiotics; these enzymes increase the production of contact allergens that induce inflammatory responses in human keratinocytes[30]. PPD is susceptible to auto-oxidation, resulting in ROS formation[31]. Contact allergic responses to PPD may result from the cytochrome P450-dependent generation of an oxidation product of PPD known as a BB[32]. The upregulation of *HMOX1*, which encodes heme oxygenase 1, was identified as a new biomarker for detecting the sensitization potential of chemicals such as PPD[33]. It was reported that PPD induces the expression and activity of two prostaglandin-endoperoxide synthases (PTGS1 and PTGS2) in human keratinocytes via oxidative processes[7]. The relative overrepresentation of PPD-upregulated genes in the GO biological process category of response to oxidative stress and the KEGG pathway of xenobiotic metabolism by cytochrome P450 supports the idea that ROS generation caused by PPD auto-oxidation, the PPD derivatives mono-acetyl-PPD and di-acetyl-PPD, or PPD-metabolizing enzymes markedly influences PPD-mediated skin toxicity, e.g., allergy.

In summary, the present genome-wide screening study demonstrated that transcriptional regulation is an important event in the cellular response to PPD exposure. In total, 1,504 genes were differentially expressed, with 854 being upregulated and 650 being downregulated, in response to PPD. We confirmed that the PPD-regulated DEGs were associated with several GO biological processes, including inflammatory and oxidative stress responses, and with several KEGG pathways, including the metabolism of xenobiotics by cytochrome P450 and the Toll-like receptor signaling pathway. Our results indicate that changes in gene expression are associated with PPD-induced toxicity, suggesting that the identified genes may be promising biomarkers for detecting PPD-induced toxicity. Further biological experiments are needed to elucidate the exact roles of these DEGs and the related pathways in this chemical-induced effect.

Materials & Methods

Cell lines and culture

The human keratinocytes HaCaT cell line was maintained under a humidified atmosphere of 5% CO_2 and 95% air at 37°C. The culture medium was DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO), penicillin, and streptomycin (GIBCO). The culture medium was refreshed every 2 to 3 days.

Cell viability

To determine cytotoxicity and effects on cell growth, an MTT [3-(4,5-dimethylthaizol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma, USA] cell proliferation assay was performed. Briefly, HaCaT cells were seeded in 24-well culture plates (BD FalconTM; Franklin Lakes, NJ, USA) at a density of 5×10^4 cells/mL. After reaching 80% confluence, the cells were exposed to Para-phenylenediamine (PPD, Sigma) for 48 h. After exposure, the cells were incubated for 3 h with 5 mg/mL MTT in phosphate-buffered saline (PBS) at 37°C. The reaction was stopped by removing the medium and adding DMSO. The absorbance of each sample was measured at 540 nm. The value of untreated sample was regarded as 100% and the 20% inhibitory concentration (IC_{20}) of cell proliferation by PPD was defined as the concentration that causes 20% reduction in the cell viability versus the untreated control. The IC_{20} values were determined directly from the linear dose-response curves. The MTT assay was performed in triplicate for each sample.

Assay for ROS generation

The rate of ROS production was assayed using 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), which reacts with ROS to form the fluorescent product

2′,7′-dichlorodihydrofluorescein (DCF). After chemicals treatment for 48 h, HaCaT cells washed twice with PBS and incubated with 20 μM DCFH-DA for 30 min. Fluorescence intensity of DCFH-DA was measured with a plate-reader fluorimeter (Fluostar Optima, BMG Labtechnologies, Offenburg, Germany) with an excitation wavelength of 485 nm and emission wavelength of 530 nm. DCF fluorescence values were normalized to total cell viability detected by MTT assay. The data were expressed as percentage of control.

RNA extraction

Total RNA was extracted from chemicals-treated HaCaT cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and was purified using an RNeasy mini kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. Genomic DNA was removed using an RNase-free DNase Set (Qiagen). Total RNA was quantitated by measuring optical absorbance (NanoDrop ND 1000 spectrophotometer; NanoDrop Technologies, Inc., Wilmington, DE, USA), and its quality was evaluated by automated gel electrophoresis (Experion™; Bio-Rad Laboratories, Hercules, CA, USA).

Microarray

Each total RNA sample (200 ng) was labeled and amplified using Low Input Quick Amp labeling kit (Agilent technologies, CA). The Cy3-labeled aRNAs were resuspended in 50 μL of hybridization solution (Agilent technologies, CA). After labeled aRNAs were placed on Agilent SurePrint G3 Human GE 8 × 60 K array (Agilent technologies, CA) and covered by a Gasket 8-plex slide (Agilent technologies, CA). The slide were hybridized for 17 hr at 65°C oven The hybridized slides were washed in 2 × SSC, 0.1% SDS for 2 min, 1 × SSC for 3 min, and then 0.2 × SSC for 2 min at room temperature. The slides were centrifuged at 3,000 rpm for 20 sec to dry.

Data analysis

The arrays were analyzed using an Agilent scanner with associated software. Gene expression levels were calculated with Feature Extraction v10.7.3.1 (Agilent technologies, CA). Relative signal intensities for each gene were generated using the Robust Multi-Array Average algorithm. The data were processed based on quantile normalization method using the GeneSpring GX 11.5.1 (Agilent technologies, CA). This normalization method aims to make the distribution of intensities for each array in a set of arrays the same. The normalized, and log transformed intensity values were then analyzed using GeneSpring GX 11.5.1 (Agilent technologies, CA). Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes.

Functional analysis

In order to classify the selected genes into groups with a similar pattern of expression, each gene was assigned to an appropriate category according to its main cellular function. Gene ontology (GO) analysis through the DAVID program (http://david.abcc.ncifcrf.gov) in order to study chemicals affected gene expression.

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