Toxicology in Vitro 27 (2013) 2213–2224

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

Development of a new *in vitro* skin sensitization assay (Epidermal Sensitization Assay; EpiSensA) using reconstructed human epidermis

Kazutoshi Saito, Yuko Nukada, Osamu Takenouchi, Masaaki Miyazawa *, Hitoshi Sakaguchi, Naohiro Nishiyama

Kao Corporation, Safety Science Research Laboratories, 2606 Akabane, Ichikai-Machi, Haga-Gun, Tochigi 321-3497, Japan

ARTICLE INFO

Article history:
Received 6 January 2013
Accepted 22 August 2013
Available online 30 August 2013

Keywords:
Skin sensitization
Alternative method
Keratinocytes
Reconstructed human epidermis (RhE) model
Microarray analysis
Gene expression

ABSTRACT

Recent changes in regulatory requirements and social views on animal testing have accelerated the development of reliable alternative tests for predicting skin sensitizing potential of chemicals. In this study, we aimed to develop a new *in vitro* skin sensitization assay using reconstructed human epidermis, RhE model, which is expected to have broader applicability domain rather than existing *in vitro* assays. Microarray analysis revealed that the expression of five genes (ATF3, DNAJB4, GCLM, HSPA6 and HSPH1) related to cellular stress response were significantly up-regulated in RhE model after 6 h treatment with representative skin sensitizers, 1-fluoro-2,4-dinitrobenzene and oxazolone, but not a non-sensitizer, benzalkonium chloride. The predictive performance of five genes was examined with eight skin sensitizers (e.g., cinnamic aldehyde), four non-sensitizers (e.g., sodium lauryl sulfate) and four pre-/pro-haptens (e.g., *p*-phenylenediamine, isoeugenol). When the positive criteria were set to obtain the highest accuracy with the animal testing (LLNA), ATF3, DNAJB4 and GCLM exhibited a high predictive accuracy (100%, 93.8% and 87.5%, respectively). All tested pre-/pro-haptens were correctly predicted by both ATF3 and DNAJB4. These results suggested that the RhE-based assay, termed epidermal sensitization assay (EpiSensA), could be an useful skin sensitization assay with a broad applicability domain including pre-/pro-haptens.

1. Introduction

Allergic contact dermatitis (ACD) resulting from skin sensitization is a common occupational and environmental health problem (Peiser et al., 2012). To date, the identification and evaluation of skin sensitizers rely on animal tests, such as the local lymph node assay (LLNA) (Kimber et al., 2002). However, the ethical issues and the European Union ban on animal testing for cosmetic ingredients (Cosmetics Regulation EC 1223/2009) have accelerated the development of *in vitro* tests for evaluating skin sensitization potential of chemicals.

ACD is an adaptive inflammatory response caused by skin sensitizers. Skin sensitizers, which penetrate through the skin, form allergen–protein complex (haptenated proteins) and are captured by dendritic cells (DCs) (Sasaki and Aiba, 2007). These DCs mature and migrate towards the draining lymph nodes, where DCs present the peptides from haptenated proteins to naive T cells. Finally, antigen-specific T cells proliferate and disseminate into the peripheral circulation (Kimber et al., 2011). Repeated exposure to same skin sensitizers after induction of sensitization can result in elicitation and symptoms of ACD (Toebak et al., 2009).

Keratinocytes (KCs) are not only the dominating cells in the epidermis where the formation of allergen-protein complex occurs, but also the first cells which encounter chemicals penetrating through stratum corneum. KCs exposed to skin sensitizers can secrete some cytokines (e.g., IL-18, TNF-α (Luster et al., 1995; Naik et al., 1999), which influence the allergen-induced DC maturation and migration from the skin to the draining lymph nodes

Abbreviations: ACD, allergic contact dermatitis; ADH, alcohol dehydrogenase; AOO, acetone: olive oil = 4:1; ATF3, activating transcription factor 3; BKC, benzalkonium chloride; CA, cinnamic aldehyde; CinA, cinnamic alcohol; CYP, cytochrome P450; DAVID, Database for Annotation, Visualization and Integrated Discovery; DCs, dendritic cells; DNAJB4, dnaJ (Hsp40) homolog, subfamily B, member 4; DNCB, 2,4-dinitrochlorobenzene; DNFB, 1-fluoro-2,4-dinitrobenzene; ECVAM, European Centre for the Validation of Alternative Methods; EpiSensA, Epidermal Sensitization Assay; ER, endoplasmic reticulum; GCLM, glutamate-cysteine ligase, modifier subunit; GO, gene ontology; GSH, glutathione; HSPA6, heat shock 70 kDa protein 6; HSPH1, heat shock 105 kDa/110 kDa protein 1; IEU, isoeugenol; IL, interleukin; KCs, keratinocytes; LA, lactic acid; LLNA, local lymph node assay; 2-MBT, 2-mercaptobenzothiasole; MDBGN, methyldibromo glutaronitrile; MTT, methylthiazolydiphenyl-tetrazolium; 4-NBB, 4-nitrobenzyl bromide; OXA, oxazolone; PPD, *p*-phenylenediamine; RhE, reconstructed human epidermis; ROS, reactive oxygen species; SA, salicylic acid; SLS, sodium lauryl sulfate; TMTD, tetramethylthiuram disulfide; TNF, tumor necrosis factor.

* Corresponding author. Tel.: +81 285 68 7342.
E-mail address: miyazawa.masaaki@kao.co.jp (M. Miyazawa).

0887-2333/$ - see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.tiv.2013.08.007

(Cumberbatch et al., 1997; Antonopoulos et al., 2008). Thus, due to their anatomical location and important roles in skin sensitization, KCs represent an important cell type to base the development of *in vitro* skin sensitization assays. In fact, several *in vitro* skin sensitization tests using KC-like cell lines (e.g., HaCaT) have been reported, focusing on the change of transcriptional activity (Emter et al., 2010), gene expression (Mckim et al., 2010; Vandebriel et al., 2010) and cytokine production (Corsini et al., 2009). Three-dimensional reconstructed human epidermis models (RhE models) consist of normal human-derived epidermal keratinocytes (NHEK), which have been cultured to form a multilayered, highly differentiated model of the human epidermis (Netzlaff et al., 2005). Since RhE models are cultured at the air–liquid interface, test chemicals can be directly applied to RhE models in a manner similar to the real skin. Moreover, it has been reported that RhE models exhibit metabolic capability similar to that in human skin (Luu-The et al., 2009; Götz et al., 2012a, 2012b). This feature indicates that RhE models could be applicable to the evaluation of pre-/pro-haptens, which form protein reactive haptens through either air-oxidation or metabolic conversion (Aptula et al., 2007). Thus, skin sensitization tests with RhE models have the potential to evaluate a wide variety of chemicals including pre-/pro-haptens with similar performance to *in vivo* tests. However, the information on specific biomarkers to detect the skin sensitization potential of chemicals in RhE models has been limited (Corsini et al., 2002; Mckim et al., 2012).

In this study, we aimed to develop a novel skin sensitization assay using a RhE model, EpiDerm™, which is commercially available model provided by MatTek Corp. (Ashland, MA, USA). EpiDerm™ is globally used for toxicological evaluations like skin permeability potential (Netzlaff et al., 2005) and genotoxicity potential (Curren et al., 2006) of chemicals as well as adopted for skin irritation and corrosion testing by the OECD as Test Guideline 439 and 431 (OECD, 2004, 2010). First, we performed microarray analysis using EpiDerm™ treated with two representative sensitizers (1-fluoro-2,4-dinitrobenzene; DNFB and oxazolone; OXA) and one non-sensitizer (benzalkonium chloride; BKC) to investigate the gene expression profile significantly induced by skin sensitizers. Then, we evaluated the predictive performance of the assay based on expression of the selected marker genes using 16 reference chemicals recommended by European Centre for the Validation of Alternative Methods (ECVAM) (Casati et al., 2009).

2. Materials and methods

2.1. Chemicals and sample preparation

The sensitizers employed in the experiment were: cinnamic alcohol (CinA), cinnamic aldehyde (CA), 2,4-dinitrochlorobenzene (DNCB), eugenol, oxazolone (OXA), 1-fluoro-2,4-dinitrobenzene (DNFB), glyoxal, isoeugenol (IEU), 2-mercaptobenzothiasole (2-MBT), methyldibromo glutaronitrile (MDBGN), 4-nitrobenzyl bromide (4-NBB), *p*-phenylenediamine (PPD), and tetramethylthiuram disulfide (TMTD). The non-sensitizers were: benzalkonium chloride (BKC), glycerol, lactic acid (LA), salicylic acid (SA), and sodium lauryl sulfate (SLS), taking into account that SLS is well known as false positive in the LLNA based on the human sensitizing potential. All tested chemicals above were purchased from Sigma–Aldrich (St. Louis, MO, USA). Tested chemicals were dissolved in AOO (acetone (Sigma–Aldrich): olive oil (Kanto chemistry, Tokyo, Japan) = 4:1) or distilled water (Otsuka Pharmaceutical Factory, Inc., Tokyo, Japan) when applied to the RhE model, since both vehicles have been often adopted in dermal application of animal testing and failed to affect cytotoxicity under testing condition in this study. AOO was used for 14 tested chemicals (BKC, CinA, CA, DNCB, DNFB, eugenol, IEU, 2-MBT, MDBGN, 4-NBB, OXA, PPD, SA and TMTD) and distilled water was used for 4 chemicals (glycerol, glyoxal, LA and SLS).

2.2. Tissue culture

The EPI-200 (24 well format) and EPI-296 (96 well format) of EpiDerm™ were used in this study. The tissues were pre-cultured overnight at 37 °C (5% CO_2) in 0.9 mL/tissue (EPI-200) or 0.25 mL/tissue (EPI-296) of culture media provided by the manufacturer.

2.3. Microarray analysis

2.3.1. Chemical exposure

To perform microarray analysis, two representative skin sensitizers, DNFB and OXA, and one non-sensitizer, BKC, were used as test chemicals. The AOO solvent was used as a vehicle. Each test chemical was applied in triplicate at one concentration (DNFB; 0.08%, OXA; 0.4% and BKC; 0.2%) showing comparable levels of cytotoxicity by MTT assay (see 2-5-2) (cell viabilities were 58% for DNFB, 48% for OXA and 56% for BKC, respectively). After pre-incubation, the EPI-200 were moved to 24 well plate filled with 0.2 mL/well of culture media and were exposed to 10 μL/tissue test chemicals on the top for 6 h at 37 °C (5% CO_2). The 6 h exposure was determined based on the previous finding that a unique set of genes encoding redox regulatory enzymes (e.g., heme oxygenase 1 and glutamate-cysteine ligase, modifier subunit; GCLM) were significantly (>2-fold, $p < 0.05$) up-regulated in mouse ear treated for 6 h with DNFB, but not with BKC (Miyazawa and Takashima, 2012). Non-treated and vehicle-treated tissues were prepared as control tissues.

2.3.2. RNA isolation

Following the 6 h treatment with chemicals in triplicate, the tissues were rinsed three times in 150 μL pre-warmed PBS(−) (provided by MatTek), gently removed, placed into 2.0 mL microtube containing 1 mL TRIzol® (Invitrogen Corp., Carlsbad, CA, USA) and homogenized by vortex mixing. 200 μL chloroform (Tokyo Chemical Industry, Tokyo, Japan) was added to each microtube with homogenized samples. The samples were centrifuged at 12,000*g* for 15 min at 4 °C. The aqueous phase containing RNA was transferred to a 1.5 mL microtube and RNA was purified using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. The RNA was quantified by a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE) stored at −70 °C until use.

2.3.3. Microarray and data analysis

Total RNA (250 ng/sample) from the RhE model was amplified, labeled by biotinylated-ribonucleotide analog, and hybridized with Affymetrix Human Genome U133 Plue 2.0 (Affymetrix Inc., Santa Clara, CA, USA) by using GeneChipR 3′ IVT Express Kit (Affymetrix Inc.). Fluorescence signals were measured using the GeneChip Scanner 3000 and digitalization was done by GeneChip Operating Software ver 1.4 with statistical algorism. The raw data was analyzed by GeneSpring™ (Version 12.0, Agilent technologies, Inc., Santa Clara, CA, USA) to compare the expression level between the test chemicals after normalization and the correction of baseline according to the procedure recommended by the manufacturer. To identify differentially expressed genes between the four different groups (AOO, DNFB, OXA, and BKC), one-way ANOVA was performed for sample group ($p < 0.05$). The genes which were significantly ($p < 0.05$) up-regulated > 1.5-fold by both DNFB and OXA but not by BKC compared to AOO, were examined in order to analyze the enrichment of functional associations (Gene Ontology (GO) biological process terms; GO terms) using Database for

Annotation, Visualization and Integrated Discovery (DAVID, http://david.abcc.ncifcrf.gov/). The genes with top-ranked GO terms (each enrichment score was over 1.3) were subjected to further analysis.

2.4. Confirmation of microarray data by real-time PCR

2.4.1. cDNA synthesis

The Superscript™ III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) was used to prepare cDNA. The mixture consisted of 1 μL of 10 mM dNTP mix, 1 μL of Oligo (dT) (0.5 μg/μL), RNase free water (above 3 reagents are supplied by manufacturer) and 0.5 μg of total RNA (variable volume) were prepared to achieve a total volume of 10 μL. The mixture was incubated at 65 °C for 5 min and then on ice for 1 min. A mixture of 10 × RT Buffer (2 μL), 25 mM $MgCl_2$ (4 μL), 0.1 M DTT (2 μL), and RNase Out (1 μL) was added to the reaction and incubated at 42 °C for 2 min. Then, 1 μL of Super Script III was added and incubated at 42 °C for 50 min. The reaction was terminated by incubation at 70 °C for 15 min. The cDNA was treated with 1 μL RNase H for 20 min at 37 °C and then stored at -20 °C.

2.4.2. Real-time PCR

The primers and probes for five marker genes (activating transcription factor 3; ATF3, DnaJ (Hsp40) homolog, subfamily B, member 4; DNAJB4, glutamate-cysteine ligase, modifier subunit; GCLM, Heat shock 70 kDa protein 6; HSPA6 and heat shock 105 kDa/110 kDa protein 1; HSPH1) and one endogenous control gene (glyceraldehyde 3-phosphate dehydrogenase; GAPDH) are designed by Assays-by-Design Service from Applied Biosystems and the sequence information remains confidential. The primers and probes were delivered as a 20× Taqman Gene Expression Assay mix (Applied Biosystems). A total volume of 20 μL sample which consisted of 1 μL TaqMan® Gene Expression Assay 20×, 10 μL TaqMan Universal PCR Master Mix (Applied Biosystems), 1 μL cDNA template and 8 μL dH_2O was prepared and applied to optical reaction plate (96 wells; Applied Biosystems). Real-time PCR reactions were performed in the Applied Biosystems 7500 Fast Real-Time PCR System (Applied Biosystems). Relative gene expression levels versus control (fold change) were calculated using the $2^{-\Delta\Delta Ct}$ method (comparative CT method; Livak and Schmittgen, 2001)

2.5. Assessment of the predictive capability of marker genes for skin sensitization

2.5.1. Chemical treatment and analysis of gene expression

A total of 16 chemicals (8 sensitizers, 4 non-sensitizers and 4 pre-/pro-haptens) were used to assess the predictive performance of the five marker genes using the RhE model. These 16 chemicals have been recommended by ECVAM as reference chemicals for development of *in vitro* skin sensitization tests (Casati et al., 2009) and contained OXA, but did not DNFB and BKC. In this experiment, we used EPI-296, a high-throughput model (96 well type) of EpiDerm™ to evaluate multiple chemicals with various concentrations. All test chemicals were dissolved in AOO or distilled water at maximum soluble concentrations and 2-fold serial dilutions were performed to prepare the test chemical solutions applied to EPI-296. A maximum of 14 test chemical solutions with dose ranges from maximum soluble concentrations to minimum 0.01% were prepared for each chemical. 10 μL of test chemical solutions were applied to the surface of EPI-296 and incubated for 6 h at 37 °C (5% CO_2). Non-treated, and vehicle-treated tissues were also prepared as a control tissue. A total of three wells were used for one tested concentration of each chemical. After 6 h treatment, two out of the three wells were used to analyze expression levels of marker genes. The remaining one well was used for cell viability measurement by MTT assay (see 2-5-2). The expression data of each gene from tissues with over 50% cell viability were used to assess the predictive performance of EpiSensA.

2.5.2. MTT assay

To determine cell viability, the MTT assay was performed. Methylthiazolydiphenyl-tetrazolium bromide (MTT, Sigma–Aldrich Co.) solution at 0.5 mg/mL in culture medium (provided by MatTek) was prepared. The wells were washed with PBS (−) three times and 300 μL or 150 μL of MTT solution was placed into a 24 well (EPI-200) or 96 well (EPI-296) plate and incubated for 3 h at 37 °C (5% CO_2). After incubation, the MTT solution was discarded and the tissues were gently removed and placed into 200 μL isopropanol (Sigma–Aldrich Co.). The extraction process was performed for 2 h at room temperature. The absorbance of the extract was measured at 570 nm with a plate reader (BMG LABTECH GmbH, Offenburg, Germany). The absorbance of the untreated control tissue exposed to culture media was set to represent 100% of viability and the results were expressed as percentage of untreated control.

3. Results

3.1. Microarray analysis and selection of marker candidate

In order to identify the biomarkers to discriminate sensitizers and non-sensitizers in the RhE model, we examined the gene expression profile with microarray after a 6 h treatment with two representative sensitizers, DNFB and OXA, and one non-sensitizer BKC. In accordance with the previous study with DNFB- and BKC-treated mouse model (Miyazawa and Takashima, 2012), we focused on the significant up-regulated expression of know genes. A total of 1178 transcripts including 870 known genes were significantly ($p < 0.05$, $n = 3$) up-regulated more than 1.5-fold by either chemical compared to the vehicle control, AOO. Among 870 genes, 349, 387, and 100 genes were up-regulated by 0.08% DNFB, 0.4% OXA, and 0.2% BKC, respectively, as shown in the Venn diagram (Fig. 1). Of the 870 genes, the three tested chemicals up-regulated the expression of 60 genes. However, the expression of 142 genes was significantly up-regulated by both DNFB and OXA, but not BKC. These 142 genes could be used as potential candidate marker genes to discriminate between skin sensitizers and non-sensitizers.

Next, we functionally categorized the 142 genes based on the GO term using the DAVID functional annotation tool (http://david.abcc.ncifcrf.gov/), which provides GO term categories with an enrichment score. Enrichment scores are used to rank overall

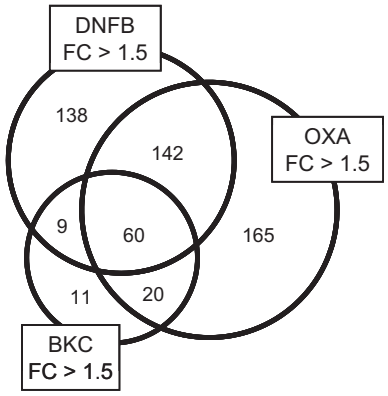

Fig. 1. Distribution of up-regulated genes by DNFB, OXA and BKC in EPI-200. The Venn diagram describes the number of genes whose expression ratio is more than 1.5-fold for at least one substances compared to vehicle control (AOO). EPI-200 was used for gene-expression microarray analysis after a 6 h treatment with two sensitizers, one non-sensitizer and AOO ($n = 3$).

importance (enrichment) of annotation terms. When a gene is associated with annotation terms with higher enrichment scores, the gene could play more important (enriched) roles (Huang et al., 2009). A total of 34 GO terms related to cell death, stress response, cellular process and metabolic processes were identified as top-ranked GO terms (enrichment scores were over 1.3) (Table 1). Positive regulation of anti-apoptosis (GO: 0045768) and response to unfolded protein (GO: 0006986) showed relatively high enrichment scores (20.7 and 7.0, respectively). Three functional categories related to negative regulation of apoptosis (GO: 0043066, GO: 0043069, and GO: 0060548) showed 2.8-fold enrichment scores. Sixty-two (62) out of the 142 genes, which were significantly up-regulated (>1.5-fold, $p < 0.05$, $n = 3$), were related to at least one category of the 34 enriched GO terms. Table 2 shows the detailed information on 62 genes; accession number, gene name, gene symbol, GO term, and fold changes induced by DNFB, OXA and BKC. When a gene is categorized into multiple GO terms, the GO term with highest enrichment score is shown in Table 2. We further set two criteria to select the promising marker genes out of these 62 genes: (1) Level of induction by either DNFB or OXA should be over 5-fold, (2) Average basal signal intensities of AOO in same marker gene should be over 100. If we follow these criteria, then DNFB up-regulated over 5-fold the expression of seven genes (HSPA6, DNAJB4, HSPH1, DHRS2, GCLM, PTX3 and FOSB), while the average basal signal intensity of AOO was more than 100 for four (HSPA6, DNAJB4, HSPH1 and GCLM) out of the seven genes (Table 2). Three of the seven genes (HSPA6, DNAJB4 and HSPH1) were related to the GO term: unfolded protein response. For OXA, two genes of the 62 genes were up-regulated over 5-fold (BRE; 5.1-fold and ATF3; 7.3-fold) and the average basal signal intensity for AOO was over 100 in only ATF3 (i.e., 129). Based on the criteria mentioned before, the five genes (ATF3, DNAJB4, GCLM, HSPA6 and HSPH1) were selected as potential marker candidates.

Table 1
GO term and enrichment scores obtained by DAVID functional annotation analysis.

GO term	Enrichment scores
GO:0045768~positive regulation of anti-apoptosis	20.7
GO:0006986~response to unfolded protein	7.0
GO:0051329~interphase of mitotic cell cycle	4.8
GO:0010629~negative regulation of gene expression	3.5
GO:0051253~negative regulation of RNA metabolic process	3.4
GO:0045934~negative regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	3.4
GO:0051172~negative regulation of nitrogen compound metabolic process	3.4
GO:0010558~negative regulation of macromolecule biosynthetic process	3.2
GO:0031327~negative regulation of cellular biosynthetic process	3.1
GO:0009890~negative regulation of biosynthetic process	3.0
GO:0043066~negative regulation of apoptosis	2.8
GO:0043069~negative regulation of programmed cell death	2.8
GO:0060548~negative regulation of cell death	2.8
GO:0031324~negative regulation of cellular metabolic process	2.6
GO:0051173~positive regulation of nitrogen compound metabolic process	2.5
GO:0045935~positive regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	2.4
GO:0009892~negative regulation of metabolic process	2.4
GO:0010605~negative regulation of macromolecule metabolic process	2.4
GO:0051254~positive regulation of RNA metabolic process	2.3
GO:0010604~positive regulation of macromolecule metabolic process	2.2
GO:0009893~positive regulation of metabolic process	2.2
GO:0031325~positive regulation of cellular metabolic process	2.1
GO:0048523~negative regulation of cellular process	2.1
GO:0048522~positive regulation of cellular process	1.9
GO:0051252~regulation of RNA metabolic process	1.7
GO:0051171~regulation of nitrogen compound metabolic process	1.7
GO:0019219~regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	1.6
GO:0031323~regulation of cellular metabolic process	1.6
GO:0031326~regulation of cellular biosynthetic process	1.5
GO:0080090~regulation of primary metabolic process	1.5
GO:0009889~regulation of biosynthetic process	1.5
GO:0060255~regulation of macromolecule metabolic process	1.5
GO:0010468~regulation of gene expression	1.5
GO:0010556~regulation of macromolecule biosynthetic process	1.4

142 genes significantly up-regulated by both DNFB and OXA but not BKC were analyzed by DAVID functional annotation tool. Highly enriched GO terms (enrichment scores > 1.3) were listed.

3.2. Confirmation of microarray data by real-time PCR

To confirm the microarray data, real-time PCR experiments were performed for the five genes. The induction levels of DNAJB4, GCLM, HSPA6, and HSPH1 genes by DNFB were 5.1-fold, 7.6-fold, 80-fold, and 5.0-fold, respectively. The induction level of ATF3 gene by OXA was 38-fold (Fig. 2). These results indicate that the over 5-fold induction of each gene observed in microarray analysis was reproduced in the real-time PCR analysis.

3.3. Assessment of predictive capability of five marker candidates for skin sensitization

The predictive performance of the selected five genes for skin sensitization was further examined in EPI-296, a high-throughput model (96 well type) of EpiDerm™. A total of 16 chemicals (8 sensitizers, 4 non-sensitizers and 4 pre-/pro-haptens) were evaluated according to the procedure described in materials and methods. These 16 reference chemicals contained OXA, but did not DNFB and BKC, which were initially tested. The viability of vehicle (AOO)-treated tissue showed 90.8 ± 7.8 (SD)% ($N = 15$).

3.3.1. Eight sensitizers and four non-sensitizers

The expression of five genes by all tested chemicals except 2-MBT and glycerol was examined at non-toxic to sub-toxic concentrations (cell viabilities were over 50%) (Fig. 3). The tissue in EPI-296 treated with 10% 2-MBT and 100% glycerol (maximum applicable concentrations) had a cell viability of 105% and 96%, respectively. All tested sensitizers induced over a 4.5-fold increase in ATF3 expression (from 4.6-fold for 0.08% MDBGN to 49.5-fold for 2.50% glyoxal) in a dose-dependent fashion (Fig. 3A). SLS also up-regulated 3.99-fold expression of ATF at 0.31% but the dose-dependency was not observed. The up-regulation of ATF3 gene expression by sensitizers was observed at non-toxic to sub-toxic concentrations, where cell viabilities were over 50% (Fig. 3A). On the other hand, none of the tested non-sensitizers induced ATF3 expression to the same extent with non-toxic to sub-toxic concentrations (Fig. 3A). For DNAJB4, all tested sensitizers induced over a 2-fold gene expression (from 2.1-fold for glyoxal to 50.7-fold for PPD) (Fig. 3B). On the other hand, the expression of DNAJB4 was also up-regulated over 2-fold (i.e., 2.2-fold) by only one concentration (0.08%) of SLS (Fig. 3B). GCLM was up-regulated over 2-fold by seven out of the eight tested sensitizers. Glyoxal induced gene expression of GCLM by 1.98-fold (Fig. 3C). All tested non-sensitizers failed to induce the expression of GCLM by more than 2-fold (Fig. 3C). HSPA6 expression varied from 2.5-fold in 0.1% OXA (where cell viability was 89%) to 120-fold in 0.39% CA with a cell viability of 62% (Fig. 3D). On the other hand, three non-sensitizers (glycerol, LA and SLS) also induced the expression of HSPA6 by

Table 2
Gene expression for 62 genes included in highly enriched GO terms.

Accession No.	Gene name	Gene symbol	GO term	Fold change[a]			Average signal of AOO[b]
				DNFB	OXA	BKC	
NM_199193	Brain and reproductive organ-expressed (TNFRSF1A modulator)	BRE		1.8	**5.1**	0.7	67
NM_001124	Adrenomedullin	ADM	GO:0045768	2.1	2.2	0.7	**4133**
NM_002184	Interleukin 6 signal transducer (gpl30, oncostatin M receptor)	IL6ST	Positive regulation of anti-apoptosis	1.7	1.8	1.2	**318**
NM_000389	Cyclin-dependent kinase inhibitor 1A	CDKN1A		1.7	1.8	1.4	**7659**
NM_002890	RAS p21 protein activator (GTPase activating protein) 1	RASA1		1.7	1.5	1.2	**734**
NM_002155	Heat shock 70 kDa protein 6	HSPA6		**48.0**	1.7	0.3	**555**
NM_007034	DnaJ (Hsp40) homolog, subfamily B, member 4	DNAJB4	GO:0006986	**5.2**	3.3	0.9	**539**
NM_006644	Heat shock 105 kDa/110 kDa protein 1	HSPH1	Response to unfolded protein	**5.8**	1.9	1.2	**1117**
NM_005345	Heat shock 70 kDa protein 1A	HSPA1A		3.1	1.6	1.1	**16006**
NM_000076	Cyclin-dependent kinase inhibitor 1C	CDKN1C		1.6	1.6	0.9	**966**
NM_001105	Activin A receptor, type I	ACVR1	GO:0051329	1.5	1.6	1.0	**781**
NM_003620	Protein phosphatase, Mg2+/Mn2+ dependent, 1D	PPM1D	Interphase of mitotic cell cycle	1.6	1.6	1.1	**853**
NM_001114171.1	FBJ murine osteosarcoma viral oncogene homolog B	FOSB		**15.5**	2.8	1.2	31
NM_025209	Enhancer of polycomb homolog 1 (Drosophila)	EPC1		4.2	1.9	1.3	96
NM_018064	Akirin 2	AKIRIN2		3.0	2.0	1.4	**708**
NM_005655	Kruppel-like factor 10	KLF10		1.6	2.8	1.2	**4058**
NM_012234	RING1 and YY1 binding protein	RYBP		1.5	2.6	0.9	**1913**
NM_006510	Tripartite motif containing 27	TRIM27	GO:0010629	2.0	2.1	1.4	**332**
NM_001145157	Nuclear receptor subfamily 2, group F, member 2	NR2F2	Negative regulation of gene expression	2.0	1.7	0.8	**507**
NM_183353	Ring finger protein, LIM domain interacting	RLIM		1.6	2.2	1.2	**441**
NM_006942	SRY (sex determining region Y)-box 15	SOX 15		1.8	1.8	1.3	**1253**
NM_001197115.1	Glutamate—cysteine ligase catalytic subunit	GCLC		2.0	1.5	1.2	**4659**
NM_005642	TAF7 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 551	TAF7		2.0	1.6	1.1	**2087**
NM_005189	Chromobox protein homolog 2	CBX2		1.6	1.6	1.0	**228**
NM_00113755	Leucine rich repeat (in FLU) interacting protein 1	LRRFIP1		1.5	1.7	1.2	**2967**
NM_005794	Dehydrogenase/reductase SDR family member 2	DHRS2	GO:0043066	**15.5**	4.2	1.2	31
NM_002061	Glutamate-cysteine ligase, modifier subunit	GCLM	Negative regulation of apoptosis	**6.8**	2.8	1.1	**948**
NM_003900	Sequestosome 1	SQSTM1		1.6	1.6	1.2	**2530**
NM_002852	Pentraxin3	PTX3		**5.6**	1.6	0.9	52
NM_002105	H2 A histone family, member X	H2AFX	GO:0051173	3.5	1.8	1.3	**1279**
NM_138394	Heterogeneous nuclear ribonucleoprotein L-like	HNRPLL	Positive regulation of nitrogen compound metabolic process	1.9	2.3	1.3	**71**
NM_001160125	Kruppel-like factor 6	KLF6		1.8	2.1	1.3	**1099**
NM_004229	Mediator complex subunit 14	MED14		1.7	1.8	0.9	66
NM_017555	Egl nine homolog 2	EGLN2	GO:0010604 Positive regulation of macromolecule metabolic process	1.9	1.7	1.3	**369**
NM_025195	Tribbles homolog 1 (Drosophila)	TRIB1		3.9	3.6	1.0	**902**
NM_002928	Regulator of G-protein signaling 16	RGS16	GO:0048523	4.7	1.9	0.7	80
NM_001184961	Paternally expressed 10	PEG10	Negative regulation of cellular process	2.6	2.6	0.9	93
NM_012406	PR domain containing 4	PRDM4		1.5	1.5	1.2	**543**
NM_001674	Activating transcription factor 3	ATF3		2.3	**7.3**	1.1	**129**
NM_021127	Phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1		3.2	3.4	1.5	**959**
NM_003463	Protein tyrosine phosphatase type IVA, member 1	PTP4A1		1.8	3.1	1.3	**1750**
NM_001554	Cysteine-rich angiogenic inducer 61	CYR61	GO:0048522	1.8	3.1	0.9	**638**
NM_003844	Tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A	Positive regulation of cellular process	2.1	2.3	1.5	**812**
NM_021649	Toll-like receptor adaptor molecule 2	TICAM2		2.2	2.1	0.9	**203**
NM_001018065	Neurotrophic tyrosine kinase receptor type 2	NTRK2		2.4	1.6	1.0	83
NM_005242	Coagulation factor II (thrombin) receptor-like 1	F2RL1		1.6	2.3	1.2	**3249**
NM_001252226	Polo-like kinase 2	PLK2		1.9	1.7	1.0	**2922**
NM_033213	Zinc finger protein 670	ZNF670		2.1	4.1	1.3	80
NM_012323	v-maf musculoaponeurotic fibrosarcoma oncogene homolog F	MAFF		2.9	3.1	1.3	**642**
NM_152603	Zinc finger protein 567	ZNF567	GO:0051252	1.7	2.6	1.2	**268**
NM_019591	Zinc finger protein 26	ZNF26	Regulation of RNA metabolic process	1.5	2.6	1.0	83

(*continued on next page*)

Table 2 (*continued*)

Accession No.	Gene name	Gene symbol	GO term	Fold change[a]			Average signal of AOO[b]
				DNFB	OXA	BKC	
NM_001880	Activating transcription factor 2	ATF2		1.7	2.2	1.1	**100**
NM_005640	Transcription initiation factor TFIID subunit 4B	TAF4B		1.6	2.2	1.1	**332**
NM_003440	Zinc finger protein 140	ZNF140		1.5	2.0	1.0	**536**
NM_007157	Zinc finger, X-linked, duplicated B	ZXDB		2.2	3.9	1.3	**118**
NM_001172	arginase, type II	ARG2	GO:0051171	2.6	2.9	1.4	**1277**
NM_145715	Tigger transposable element derived 2	TIGD2	Regulation of nitrogen compound metabolic process	1.9	3.6	1.3	**157**
NM_182972	Interferon regulatory factor 2 binding protein 2	IRF2BP2		1.8	2.0	1.1	**5494**
NM_024620	Zinc finger protein 329	ZNF329		1.8	1.7	1.0	**336**
NM_057749	Cyclm E2	CCNE2	GO:0031323	2.8	1.8	0.9	**366**
NM_005801	Eukaryotic translation initiation factor 1	EIF1	Regulation of cellular metabolic process	2.0	1.9	1.2	**8728**
NM_002577	p21 protein (Cdc42/Rac)-activated kinase 2	PAK2		1.5	2.0	1.3	**293**

[a,b] Bold letters indicate that (a) average signal values for AOO were over 100 and (b) over 5-fold increase in gene expression change.

10.4-fold with 0.01% glycerol, 39-fold with 3.13% LA, and 9.3-fold with 0.02% SLS, respectively (Fig. 3A and D). Expression of HSPH1 was up-regulated over 2-fold by glyoxal (i.e., 2.02-fold increase) and CA (i.e., 5.14-fold), while OXA, MDBGN and 2-MBT induced the expression of HSPH1 at a maximum of 1.3-fold, 1.8-fold and 1.9-fold, respectively (Fig. 3E). For two non-sensitizers, LA and SLS, up-regulated HSPH1 expression was observed up to 5.0-fold with 3.13% LA and up to 2.5-fold with 0.01% SLS (Fig. 3E).

3.3.2. Pre-/pro-haptens (PPD, IEU, eugenol and CinA)

The expression of five genes by four tested pre-/pro-haptens was examined at non-toxic to sub-toxic concentrations (cell viabilities were over 50%) (Fig. 4). The cell viabilities at 6.25% IEU, 3.13% eugenol, and 6.25% CinA were less than 50% but at 5% (maximum applied concentration) PPD was 74.7%. All tested pre-/pro-haptens induced over a 10-fold increase in ATF3 and DNAJB4 expression in a dose-dependent fashion (Fig. 4A and B). On the other hand, the expression of GCLM was up-regulated over 2-fold by three out of the four tested pre-/pro-haptens (14-fold with 0.31% PPD, 2.6-fold with 1.56% IEU and 8.5-fold with 1.56% CinA), while eugenol had a less than 2-fold increase (1.65-fold) at any tested concentration (Fig. 4C). HSPA6 expression varied from 9.0-fold with 1.56% CinA to 110-fold with 3.13% IEU (Fig. 4D). Expression of HSPH1 was up-regulated over 2-fold by all tested pre-/pro-haptens (8.2-fold with 1.25% PPD, 12-fold with 3.13% IEU and 1.57% eugenol, and 2.3-fold with 3.13% CinA) (Fig. 4E).

3.3.3. Predictive performance

Based on the gene expression data from a multi-dose study with 16 chemicals, we set a 4-fold increase in gene expression as the positive criteria for ATF3 and calculated the sensitivity, specificity, and accuracy (Table 3). ATF3 showed complete agreement with LLNA results, indicating this gene could have high predictive performance of skin sensitizers. Likewise, we set a 2-fold gene expression increase as the positive criteria for genes DNAJB4, GCLM, and HSPA6. DNAJB4, GCLM, and HSPA6 showed relatively high accuracy (93.8%, 87.5% and 81.3%, respectively), whereas the specificity of HSPA6 was relatively low (25%). GCLM showed an specificity of 100%, suggesting that GCLM might be a more sensitive marker gene to skin sensitizers. HSPH1 showed a sensitivity of 75%, specificity of 50%, and an accuracy of 68.8%, indicating that predictive performance of this gene might be lower. Regarding four tested pre-/pro-haptens (PPD, IEU, eugenol and CinA), three pre/pro-haptens (PPD, IEU and CinA) were correctly

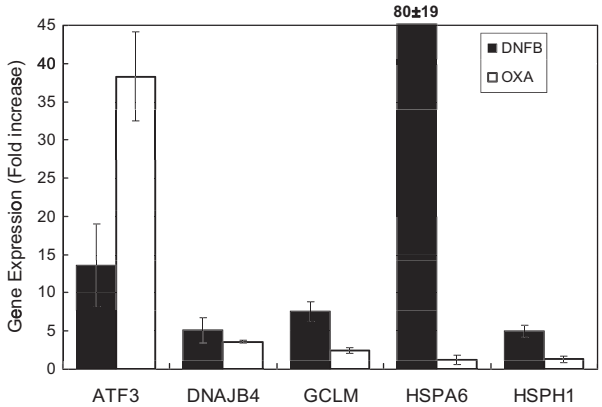

Fig. 2. Real-time PCR analyses of 5 marker candidates. EPI-200 was exposed to 0.08% DNFB or 0.4% OXA for 6 h; then the gene expression was analyzed by real-time PCR. Fold increase for five selected genes compared to vehicle control (AOO) is shown. Results are expressed as mean ± SD (n = 3).

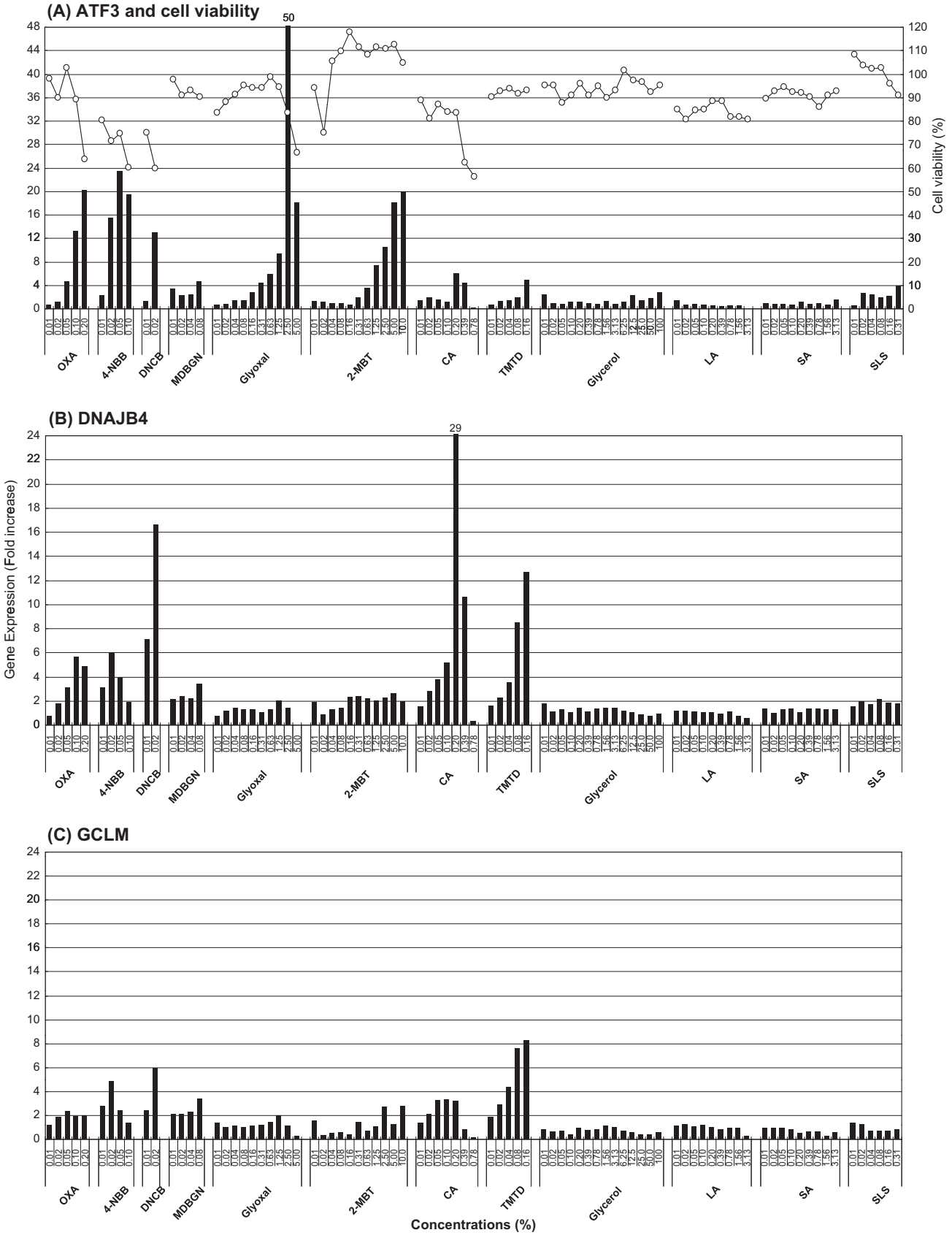

Fig. 3. Marker gene expression and cell viability for eight sensitizers and four non-sensitizers. EPI-296 was exposed to 16 reference chemicals for 6 h at indicated concentrations; then marker gene expression and cell viability were analyzed by real-time PCR and MTT assay, respectively. Gene expression increases for 5 selected genes (ATF3 (A), DNAJB4 (B), GCLM (C), HSPA6 (D) and HSPH1 (E)), compared to vehicle control (AOO or distilled water), are shown for 12 chemicals except four pre-/pro-haptens as black bars. Results are expressed as mean (n = 2). Cell viabilities were shown as outline dots (A) (n = 1). The data with over 50% of cell viability were shown for each chemical.

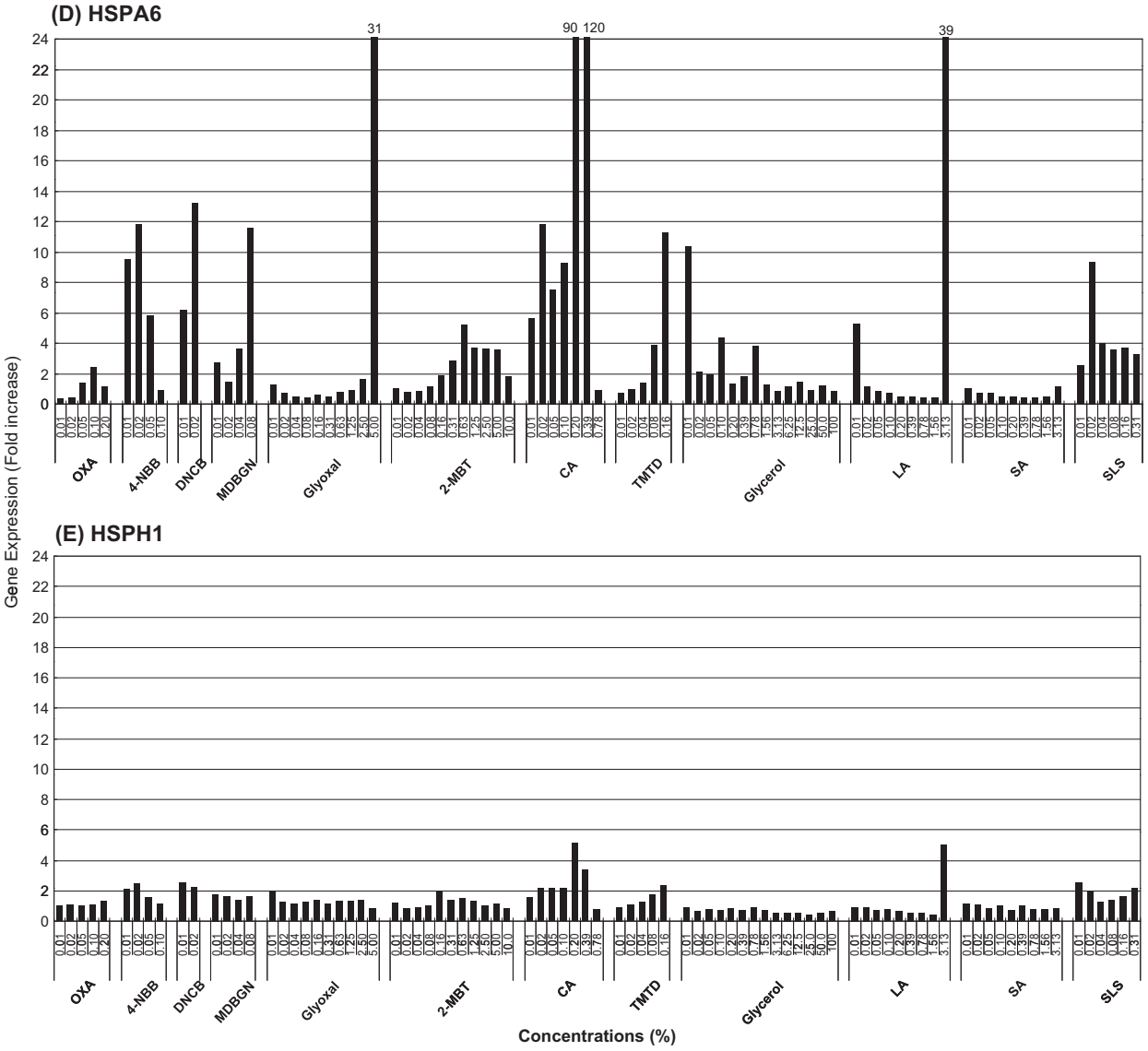

Fig. 3 (*continued*)

detected by all five marker genes and eugenol was judged as positive by four out of the five marker genes (ATF3, DNAJB4, HSPA6 and HSPH1) (Table 3).

4. Discussions

In this study, we investigated the genetic marker candidates that would detect skin sensitizers and assessed the predictive performance of these marker genes in order to develop a novel skin sensitization assay using a RhE model, EpiDerm™. Microarray analysis demonstrated that a total of 142 genes were significantly up-regulated by two sensitizers (DNFB and OXA) but not by a non-sensitizer (BKC). Furthermore, the DAVID functional analysis revealed 62 genes related to at least either one of highly enriched 34 GO terms. Then, five genes (ATF3, DNAJB4, GCLM, HSPA6, and HSPH1) out of these 62 genes were selected as marker gene candidates, taking into account the significant (>5-fold) induction level by sensitizers. When the predictive performance of the five genes was assessed with 8 sensitizers, 4 non-sensitizers, and 4 pre-/pro-haptens, ATF3 showed complete agreement with the results from an *in vivo* skin sensitization test (LLNA). DNAJB4 and GCLM also showed relatively high predictive performance, where the accuracy to LLNA was 93.8% and 87.5%, respectively. These data suggested that ATF3, DNAJB4, and GCLM could be useful markers of skin sensitization in the RhE model.

Regarding the 142 genes induced by both DNFB and OXA, and not by BKC (Fig. 1), the functional analysis with DAVID revealed that a unique set of genes related to anti-apoptosis (e.g., positive regulation of anti-apoptosis, negative regulation of apoptosis) and cellular stress response (response to unfolded protein response) were highly enriched (Table 1). In general, protein folding is induced in the endoplasmic reticulum (ER). But, several stresses, referred to ER stress, are known to reduce the protein folding capacity of the ER, which results in the accumulation of unfolded proteins in the ER and leads to apoptosis (Szegezdi et al., 2006; Ma and Hendershot, 2004). Furthermore, it has been reported that a skin sensitizer, diphenylcyclopropene, acts as ER stress inducer by up-regulating spliced XBP1 mRNA expression in human monocytic cells (Hirota et al., 2010). Taken together, the results of the functional analysis with DAVID suggested that the cytoprotective systems to survive from the stress by sensitizers were activated. In fact, DNAJB4, GCLM, HSPA6, and HSPH1 marker genes were highly associated with anti-apoptosis regulation or unfolded

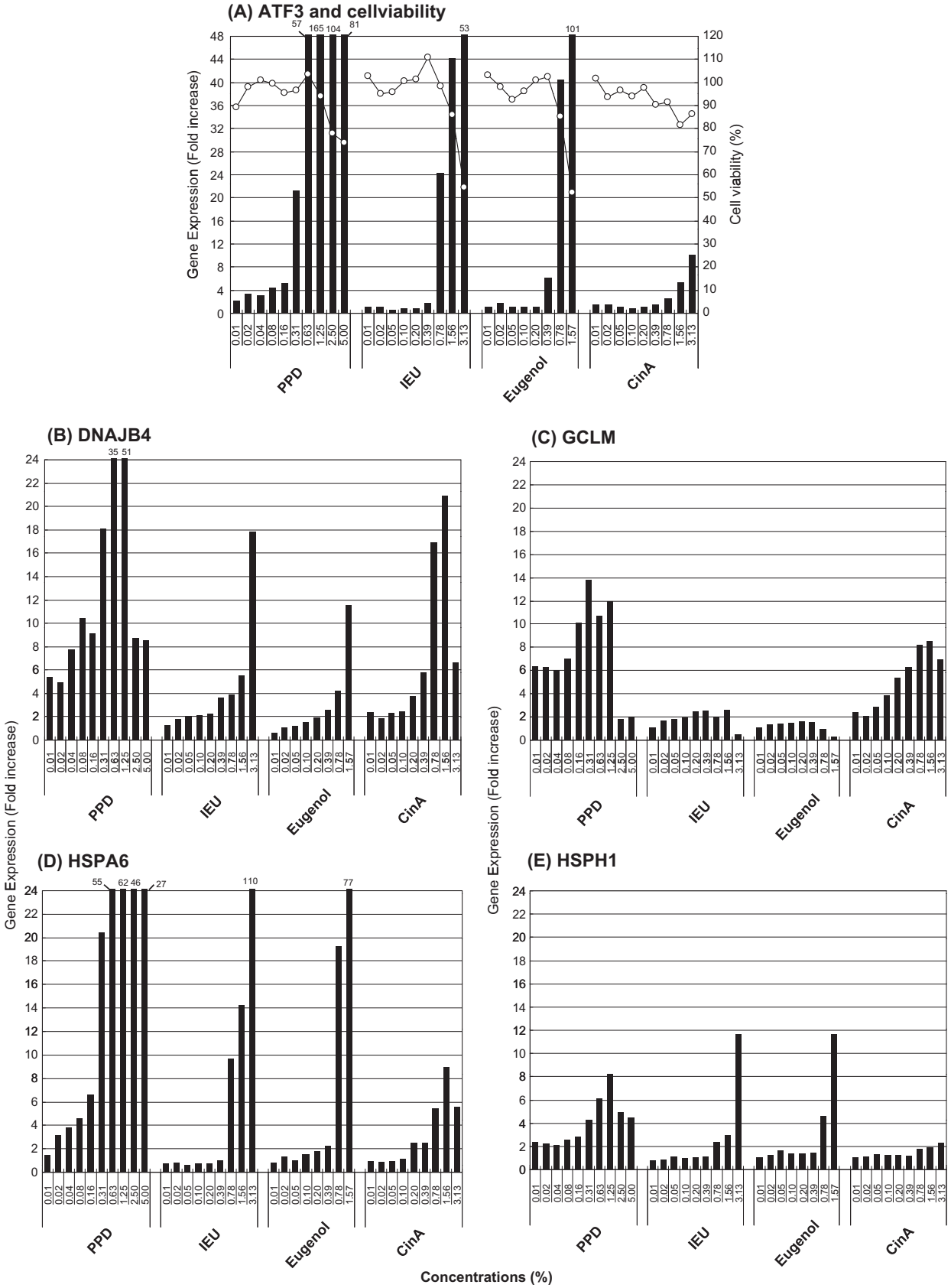

Fig. 4. Marker gene expression and cell viability for four pre-/pro-haptens. EPI-296 was exposed to chemicals for 6 h at indicated concentrations; then marker gene expression and cell viability were analyzed by real-time PCR and MTT assay, respectively. Gene expression increases for 5 selected genes (ATF3 (A), DNAJB4 (B), GCLM (C), HSPA6 (D) and HSPH1 (E)), compared to vehicle control (AOO or distilled water), are shown as black bars. Results are expressed as mean ($n = 2$). Cell viabilities were shown as outline dots (A) ($n = 1$). The data with over 50% of cell viability were shown for each chemical.

protein response. The biological relevance of five gene markers to the skin sensitization or related biological processes (e.g., keratinocyte activation, skin inflammation and immune response) is discussed below.

ATF3 is a member of the ATF/cyclic AMP response element-binding protein family of transcription factors that regulates various biological processes like immune response, apoptosis, and oncogenesis (Thompson et al., 2009). The up-regulation of ATF3 expression was observed in the lung of ovalbumin-treated wild-type mice and the ovalbumin-induced inflammatory responses (e.g., cytokine induction) markedly increased in ATF3 null-mice (Gilchrist et al., 2008); indicating that ATF3 might attenuate the allergen-induced inflammatory responses. The expression of ATF3 has been found to be also up-regulated in the epidermis of patients with skin inflammatory diseases (e.g., **erythema multiform and psoriasis**) (Pollack et al., 2010). The expression of ATF3 are also mediated by Nrf2-ARE signaling pathway (Kim et al., 2010), which regulates the expression of redox-related genes to reduce cellular stresses. Recent studies demonstrated that a lot of skin sensitizers activated Nrf2-ARE pathway in keratinocytes (Emter et al., 2010; McKim et al., 2010). Taken together, the ATF3 gene expression in epidermis or keratinocytes might lead to regulation of inflammatory responses relevant to skin sensitization.

DNAJB4, also known as HLJ1, belongs to the DNAJ/Hsp40 (heat shock protein 40) superfamily and acts as a molecular chaperone (Qiu et al., 2006). Tumor suppressive effect has been reported as a function of HLJ1 (Tsai et al., 2006), but the function of DNAJB4 in skin sensitization has not been reported. On the other hand, HSPA6 and HSPH1 also act as molecular chaperones and are members of HSPA and HSPH families, respectively. These two HSP families are highly homologous and play a prime role in protein homeostasis by protein unfolding or degradation (Vos et al., 2008). Although direct functional association of HSPA6 and HSPH1 with skin sensitization has not been reported, Yusuf et al. (2009) showed that HSP27 (HSPB) protein itself augmented DNFB-induced skin sensitization response through a Toll-like receptor 4 (TLR4)-dependent process in mouse model. Other investigators also reported that HSP70 (HSPA) induced the production of TNF-α and IL-1β, and the expression of CD86 in human DCs (Asea et al., 2002). These cytokines and the co-stimulatory molecule play important roles in the activation and antigen presentation of DCs in the early phase of skin sensitization, respectively (Toebak et al., 2009). Taking the above findings together, the HSPA6, HSPH1, and perhaps DNAJB4 might play a role in the initiation of skin sensitization.

GCLM is one of the components of glutamate cysteine ligase, which is a heterodimeric protein complex and a first rate-limiting enzyme of glutathione (GSH) synthesis (Franklin et al., 2009). This means that cellular GSH increases coupled with the up-regulation of GCLM protein level. On the other hand, Na et al. (2007) reported that contact hypersensitivity induced by skin sensitizer, trinitrocholorobenzene, was inhibited in transgenic mice over-expressing extracellular superoxide dismutase. Along with GSH, superoxide dismutase is one of the major enzymes that protect tissue from the toxic effect of reactive oxygen species (ROS) as a ROS scavenger. Considering the recent findings that skin sensitizers have potential to induce intracellular ROS production (Byamba et al., 2010; Nukada et al., 2011), it's suggested that GCLM would play a cytoprotective role from the stress induced by sensitizers in the induction of skin sensitization.

We performed the real-time PCR analysis for the five genes (ATF3, DNAJB4, GCLM, HSPA6 and HSPH1), which were significantly induced by two sensitizers in the microarray analysis. When we compared the expression of each gene induced by a sensitizer, a good correlation was found between the microarray and real-time PCR (Table 2, Fig. 2). The direction of the change (up-regulation) was consistent in all five genes. Furthermore, the magnitude of up-regulation in gene expression values in real-time PCR was quite similar in DNAJB4, GCLM, HSPA6 and HSPH1. For example, DNAJB4 expression in DNFB- and OXA-treated RhE model were up-regulated 5.2-fold (DNFB) and 3.6-fold (OXA) using the microarray, and 5.1-fold (DNFB) and 3.3-fold (OXA) using the real-time PCR. Other investigators also reported a good correlation between affymetrix microarray and real-time PCR, although minor discordances have been observed (Ryan et al., 2004). In contrast, ATF3 showed a larger gene expression increase value in the real-time

Table 3
Maximum gene expression change values and predictive performance of 5 marker genes.

Chemical name	LLNA EC3 (%)	ATF3			DNAJB4			GCLM			HSPA6			HSPH1		
		1st	2nd	mean	1st	2nd	mean	1st	2nd	mean	1st	2nd	mean	1st	2nd	mean
Oxazolone	**0.003**	15.8	24.6	**20.2**	5.73	5.64	**5.68**	1.92	2.77	**2.35**	2.13	2.83	**2.48**	1.05	1.61	1.33
4-Nitrobenzylbromide	**0.05**	17.2	29.8	**23.5**	5.38	6.47	**5.92**	6.56	3.11	**4.84**	8.22	15.3	**11.8**	1.87	3.14	**2.50**
2,4-Dinitrochlorobenzene	**0.06**	2.64	23.5	**13.1**	25.4	7.85	**16.6**	10.1	1.82	**5.96**	6.81	19.6	**13.2**	3.83	1.20	**2.52**
Methyldibromo glutaronitrile	**0.9**	5.96	3.33	**4.64**	5.18	1.68	**3.43**	4.88	1.97	**3.43**	17.1	6.07	**11.6**	2.30	1.28	1.79
Glyoxal	**1.4**	70.5	28.6	**49.5**	1.60	2.52	**2.06**	1.88	2.08	**1.98**	51.7	10.2	**31.0**	2.37	1.67	**2.02**
2-Mercaptobenzothiazole	**1.7**	23.5	16.3	**19.9**	3.15	2.02	**2.58**	4.92	0.63	**2.78**	6.73	3.62	**5.17**	1.04	2.78	1.91
Cinnamic aldehyde	**3**	5.59	6.62	**6.10**	19.8	38.9	**29.4**	3.15	3.52	**3.34**	215	25.8	**120**	4.50	5.79	**5.14**
Tetreamethylthiuram disulfide	**5.2**	8.62	1.39	**5.00**	17.9	7.53	**12.7**	7.62	8.89	**8.26**	17.7	4.90	**11.3**	2.35	2.31	**2.33**
p-Phenylenediamine[a]	**0.16**	95.8	234	**165**	38.1	63.4	**50.7**	10.3	17.3	**13.8**	57.2	66.2	**61.7**	5.66	10.76	**8.21**
Isoeugenol[a]	**1.2**	82.2	24.5	**53.4**	32.6	3.10	**17.9**	2.78	2.37	**2.57**	118	103	**110.3**	13.65	9.54	**11.6**
Eugenol[a]	**13**	57	146	**101**	4.21	18.9	**11.5**	1.16	2.13	**1.65**	22.3	132	**77.1**	5.37	17.89	**11.6**
Cinnamic alcohol[a]	**21**	2.03	18.0	**10.0**	23.8	18.0	**20.9**	7.82	9.20	**8.51**	7.11	10.8	**8.97**	1.12	3.39	**2.25**
Glycerol	–	2.24	3.46	2.85	1.64	1.90	1.77	1.27	0.99	1.13	10.6	10.2	**10.40**	1.04	0.87	0.95
Lactic acid	–	1.08	1.86	1.47	1.15	1.31	1.23	0.79	1.79	1.29	57.3	20.7	**39.0**	5.88	4.20	**5.04**
Salicylic acid	–	1.39	1.71	1.55	1.81	0.90	1.36	0.79	1.13	0.96	1.05	1.19	1.12	0.59	1.62	1.11
Sodium lauryl sulfate	–	2.46	5.51	3.99	1.91	2.40	**2.16**	1.08	1.61	1.34	11.8	6.81	**9.32**	2.63	2.40	**2.52**
Positive criteria		4-Fold			2-Fold			2-Fold			2-Fold			2-Fold		
Sensitivity (%)		100			100			83.3			100			75		
Specificity (%)		100			75			100			25			50		
Accuracy (°/o)		100			93.8			87.5			81.3			68.8		

For maximum gene expression values, both values of two replicates and the mean values were shown. The values shown in bold letters were over threshold.
[a] Pre-/pro-haptens.

PCR (DNFB; 13.6-fold, OXA; 38.3-fold) than in the microarray (DNFB; 2.3-fold, OXA; 7.3-fold). Gene expression increase measured with the real-time PCR has been reported to be higher than those measurements with microarray analysis (Szameit et al., 2008). The real time PCR studies confirmed that the selection of marker genes based on our microarray data was appropriate.

ATF3 correctly predicted the skin sensitizing potential of all tested chemicals (the accuracy was 100%), although SLS might be rated borderline. In addition, DNAJB4 and GCLM also showed high predictive performance (the accuracy was 93.8% and 87.5%, respectively) (Table 3). ATF3 and GCLM have been reported to be ARE-dependent genes (Erickson et al., 2002; Kim et al., 2010). Moreover, Emter et al. (2010) reported that the ARE reporter assay using a genetically modified HaCaT cell line, referred to KeratinoSens, provided an accuracy of over 80% to the LLNA regarding the 67 tested chemicals. Mckim et al. (2010) also showed that the combination of chemical reactivity potential and expression changes of ARE-dependent genes in the HaCaT offered an accuracy of 84% to the LLNA regarding 58 chemicals. These findings indicate that ARE-dependent genes such as ATF3 and GCLM can be sensitive markers in not only the HaCaT but also the RhE model. However, the sensitivity may vary depending on gene and chemical. In looking at the ARE genes, the induction levels of ATF3 and GCLM expression were different for the same tested sensitizer (Table 3). For example, the maximum gene expression change for ATF3 induced by eugenol (101-fold) and glyoxal (49.5-fold) were quite different from that of GCLM (1.98-fold by eugenol and 1.65-fold by glyoxal) (Table 3). Consistent with our findings, differences in magnitude of gene expression have been reported for ARE-dependent genes depending on tested sensitizers (Ade et al., 2009; Mckim et al., 2010). Other signaling pathways other than Nrf2/ARE might contribute to the difference in the expression between these genes (Inoue et al., 2004; Yang et al., 2005). Taken together, our results not only demonstrated that ATF3 and GCLM in the RhE model could be promising gene markers to predict skin sensitizing potential of chemicals, but also suggested that the combination of marker genes might be required to correctly evaluate a broad number of skin sensitizers.

Two HSP genes (HSPA6 and HSPH1) showed relatively low accuracy (HSPA6; 81.3%, HSPH1; 68.8%) compared with ATF3, DNAJB4 or GCLM. Both HSPA6 and HSPH1 expression was markedly induced by non-sensitizers, LA and SLS. The protein level of HSP27, which belongs to HSP family, has been reported to be up-regulated by some skin irritants (nonanoic acid and SLS) in human skin and/or excised skin (Boxman et al., 2002). A second investigator reported that the expression of HSPA1 gene was up-regulated in SLS-treated RhE model (Niwa et al., 2009). Although the precise mechanism for which SLS or other irritants up-regulate the gene expression of HSPs has remained unknown, these findings suggest that some HSP-related genes might not be suitable markers to detect skin sensitizers.

ATF3 and DNAJB4 correctly detected all tested pre-/pro-haptens (PPD, IEU, eugenol and CinA) (Table 3). It has been reported that the correct prediction of pre-/pro-haptens in *in vitro* tests is challenging. For instance, isoeugenol was not detected as a sensitizer in the "human Cell Line Activation Test", which focuses on the change in expression level of cell surface molecules (CD86 and CD54) in DC-like cell line THP-1 cells (Ashikaga et al., 2010). Eugenol was not correctly predicted in the KeratinoSens (Emter et al., 2010). The authors of these studies have suggested that false outcomes might attribute to the lack of metabolic enzymes like cytochrome P450 (CYP), which has been suggested to play a role in the bioactivation of pro-haptens (Chipinda et al., 2011). On the other hand, Gerberick et al. (2009) incorporated a peroxidase system into the "direct peptide reactivity assay", an *in chemico* method based on the reactivity to model peptides, to detect pre-/pro-haptens. Their findings showed that the addition of peroxidase system dramatically improved the reactivity of eugenol, but not CinA, to a model peptide (Gerberick et al., 2009). CinA is a pro-hapten which could be converted to CA, a complete hapten, by alcohol dehydrogenase (ADH) (Elahi et al., 2004). EpiDerm™ and human skin have been reported to display similar gene expression profiles of drug metabolizing enzymes including CYP and ADH (Hu et al., 2010; van Eijl et al., 2012). With regard to their enzymatic activities, Götz et al. (2012) demonstrated that the basal CYP enzymatic activities were very low in both EpiDerm™ and human skin, but the CYP-mediated biotransformation in EpiDerm™ was significantly enhanced after treatment with specific inducers. Moreover, Jäckh et al. (2011) characterized the basal activities of Flavin-dependent monooxygenases, *N*-acetyltransferases, and UDP-glucuronyltransferases in EpiDerm™. Thus, it is possible that *in vivo*-like expression of drug metabolizing enzymes in the RhE model may contribute the detection of pre-/pro-haptens.

In summary, we developed the RhE-based assay, termed epidermal sensitization assay (EpiSensA) for detecting skin sensitizers by measuring the expression of ATF3, DNAJB4, and GCLM genes. This assay correctly predicted the 16 reference chemicals recommended by ECVAM including pre-/pro-haptens. To further examine the utility of EpiSensA as a part of test battery for non-animal skin sensitization assessment, we would need to not only increase the number of tested chemicals but also test the chemicals which are difficult to be evaluated in cell culture systems due to high lipophilicity.

Conflict of interest

None declared.

Acknowledgement

We thank Dr. Javier Avalos for his critical review of the manuscript.

References

Ade, N., Leon, F., Pallardy, M., Peiffer, J.L., Kerdine-Romer, S., Tissier, M.H., Bonnet, P.A., Fabre, I., Ourlin, J.C., 2009. HMOX1 and NQO1 genes are upregulated in response to contact sensitizers in dendritic cells and THP-1 cell line: role of the Keap1/Nrf2 pathway. Toxicol. Sci. 107, 451–460.

Antonopoulos, C., Cumberbatch, M., Mee, J.B., Dearman, R.J., Wei, X.Q., Liew, F.Y., Kimber, I., Groves, R.W., 2008. IL-18 is a key proximal mediator of contact hypersensitivity and allergen-induced Langerhans cell migration in murine epidermis. J. Leukoc. Biol. 83, 361–367.

Aptula, A.O., Roberts, D.W., Pease, C.K., 2007. Haptens, prohaptens and prehaptens, or electrophiles and proelectrophiles. Contact Dermatitis 56, 54–56.

Asea, A., Rehli, M., Kabingu, E., Boch, J.A., Bare, O., Auron, P.E., Stevenson, M.A., Calderwood, S.K., 2002. Novel signal transduction pathway utilized by extracellular HSP70: role of toll-like receptor (TLR) 2 and TLR4. J. Biol. Chem. 277, 15028–15034.

Ashikaga, T., Sakaguchi, H., Sono, S., Kosaka, N., Ishikawa, M., Nukada, Y., Miyazawa, M., Ito, Y., Nishiyama, N., Itagaki, H., 2010. A comparative evaluation of in vitro skin sensitisation tests: the human cell-line activation test (h-CLAT) versus the local lymph node assay (LLNA). Altern. Lab. Anim. 38, 275–284.

Boxman, I.L., Hensbergen, P.J., Van Der Schors, R.C., Bruynzeel, D.P., Tensen, C.P., Ponec, M., 2002. Proteomic analysis of skin irritation reveals the induction of HSP27 by sodium lauryl sulphate in human skin. Br. J. Dermatol. 146, 777–785.

Byamba, D., Kim, T.G., Kim, D.H., Je, J.H., Lee, M.G., 2010. The roles of reactive oxygen species produced by contact allergens and irritants in monocyte-derived dendritic cells. Ann. Dermatol. 22, 269–278.

Casati, S., Aeby, P., Kimber, I., Maxwell, G., Ovigne, J.M., Roggen, E., Rovida, C., Tosti, L., Basketter, D., 2009. Selection of chemicals for the development and evaluation of *in vitro* methods for skin sensitization testing. Altern. Lab. Anim. 37, 305–312.

Chipinda, I., Hettick, J.M., Siegel, P.D., 2011. Haptenation: chemical reactivity and protein binding. J. Allergy (Cairo) 2011, 836982.

Corsini, E., Sheasgreen, J., Marinovich, M., Galli, C.L., 2002. Use of differential display-polymerase chain reaction to identify genes selectively modulated by chemical allergens in reconstituted human epidermis. Toxicol. In Vitro 16, 427–431.

Corsini, E., Mitjans, M., Galbiati, V., Lucchi, L., Galli, C.L., Marinovich, M., 2009. Use of IL-18 production in a human keratinocyte cell line to discriminate contact sensitizers from irritants and low molecular weight respiratory allergens. Toxicol. In Vitro 23, 789–796.

Cumberbatch, M., Dearman, R.J., Kimber, I., 1997. Langerhans cells require signals from both tumour necrosis factor-alpha and interleukin-1 beta for migration. Immunology 92, 388–395.

Curren, R.D., Mun, G.C., Gibson, D.P., Aardema, M.J., 2006. Development of a method for assessing micronucleus induction in a 3D human skin model (EpiDerm). Mutat. Res. 607, 192–204.

Elahi, E.N., Wright, Z., Hinselwood, D., Hotchkiss, S.A., Basketter, D.A., Pease, C.K., 2004. Protein binding and metabolism influence the relative skin sensitization potential of cinnamic compounds. Chem. Res. Toxicol. 17, 301–310.

Emter, R., Ellis, G., Natsch, A., 2010. Performance of a novel keratinocyte-based reporter cell line to screen skin sensitizers *in vitro*. Toxicol. Appl. Pharmacol. 245, 281–290.

Erickson, A.M., Nevarea, Z., Gipp, J.J., Mulcahy, R.T., 2002. Identification of a variant antioxidant response element in the promoter of the human glutamate-cysteine ligase modifier subunit gene. Revision of the ARE consensus sequence. J. Biol. Chem. 277, 30730–30737.

Franklin, C.C., Backos, D.S., Mohar, I., White, C.C., Forman, H.J., Kavanagh, T.J., 2009. Structure, function, and post-translational regulation of the catalytic and modifier subunits of glutamate cysteine ligase. Mol. Aspects Med. 30, 86–98.

Gerberick, G.F., Troutman, J.A., Foertsch, L.M., Vassallo, J.D., Quijano, M., Dobson, R.L., Goebel, C., Lepoittevin, J.P., 2009. Investigation of peptide reactivity of pro-hapten skin sensitizers using a peroxidase-peroxide oxidation system. Toxicol. Sci. 112, 164–174.

Gilchrist, M., Henderson Jr, W.R., Clark, A.E., Simmons, R.M., Ye, X., Smith, K.D., Aderem, A., 2008. Activating transcription factor 3 is a negative regulator of allergic pulmonary inflammation. J. Exp. Med. 205, 2349–2357.

Götz, C., Pfeiffer, R., Tigges, J., Blatz, V., Jäckh, C., Freytag, E.M., Fabian, E., Landsiedel, R., Merk, H.F., Krutmann, J., Edwards, R.J., Pease, C., Goebel, C., Hewitt, N., Fritsche, E., 2012a. Xenobiotic metabolism capacities of human skin in comparison with a 3D epidermis model and keratinocyte-based cell culture as *in vitro* alternatives for chemical testing: activating enzymes (Phase I). Exp. Dermatol. 21, 358–363.

Götz, C., Pfeiffer, R., Tigges, J., Ruwiedel, K., Hübenthal, U., Merk, H.F., Krutmann, J., Edwards, R.J., Abel, J., Pease, C., Goebel, C., Hewitt, N., Fritsche, E., 2012b. Xenobiotic metabolism capacities of human skin in comparison with a 3D-epidermis model and keratinocyte-based cell culture as *in vitro* alternatives for chemical testing: phase II enzymes. Exp. Dermatol. 21, 364–369.

Hirota, M., Motoyama, A., Suzuki, M., Yanagi, M., Kitagaki, M., Kouzuki, H., Hagino, S., Itagaki, H., Sasa, H., Kagatani, S., Aiba, S., 2010. Changes of cell-surface thiols and intracellular signaling in human monocytic cell line THP-1 treated with diphenylcyclopropenone. J. Toxicol. Sci. 35, 871–879.

Hu, T., Khambatta, Z.S., Hayden, P.J., Bolmarcich, J., Binder, R.L., Robinson, M.K., Carr, G.J., Tiesman, J.P., Jarrold, B.B., Osborne, R., Reichling, T.D., Nemeth, S.T., Aardema, M.J., 2010. Xenobiotic metabolism gene expression in the EpiDerm *in vitro* 3D human epidermis model compared to human skin. Toxicol. In Vitro 24, 1450–1463.

Huang da, W., Sherman, B.T., Lempicki, R.A., 2009. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nat. Protoc. 4, 44–57.

Inoue, K., Zama, T., Kamimoto, T., Aoki, R., Ikeda, Y., Kimura, H., Hagiwara, M., 2004. TNFalpha-induced ATF3 expression is bidirectionally regulated by the JNK and ERK pathways in vascular endothelial cells. Genes Cells 9, 59–70.

Jäckh, C., Blatz, V., Fabian, E., Guth, K., van Ravenzwaay, B., Reisinger, K., Landsiedel, R., 2011. Characterization of enzyme activities of Cytochrome P450 enzymes, Flavin-dependent monooxygenases, N-acetyltransferases and UDP-glucuronyltransferases in human reconstructed epidermis and full-thickness skin models. Toxicol. In Vitro 25, 1209–1214.

Kim, K.H., Jeong, J.Y., Surh, Y.J., Kim, K.W., 2010. Expression of stress-response ATF3 is mediated by Nrf2 in astrocytes. Nucl. Acids Res. 38, 48–59.

Kimber, I., Dearman, R.J., Basketter, D.A., Ryan, C.A., Gerberick, G.F., 2002. The local lymph node assay: past, present and future. Contact Dermatitis 47, 315–328.

Kimber, I., Basketter, D.A., Gerberick, G.F., Ryan, C.A., Dearman, R.J., 2011. Chemical allergy: translating biology into hazard characterization. Toxicol. Sci. 120 (Suppl. 1), S238–S268.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25, 402–408.

Luster, M.I., Wilmer, J.L., Germolec, D.R., Spalding, J., Yoshida, T., Gaido, K., Simeonova, P.P., Burleson, F.G., Bruccoleri, A., 1995. Role of keratinocyte-derived cytokines in chemical toxicity. Toxicol. Lett. 82–83, 471–476.

Luu-The, V., Duche, D., Ferraris, C., Meunier, J.R., Leclaire, J., Labrie, F., 2009. Expression profiles of phases 1 and 2 metabolizing enzymes in human skin and the reconstructed skin models Episkin and full thickness model from Episkin. J. Steroid Biochem. Mol. Biol. 116, 178–186.

Ma, Y., Hendershot, L.M., 2004. The role of the unfolded protein response in tumour development: friend or foe? Nat. Rev. Cancer 4, 966–977.

McKim Jr, J.M., Keller 3rd, D.J., Gorski, J.R., 2010. A new *in vitro* method for identifying chemical sensitizers combining peptide binding with ARE/EpRE-mediated gene expression in human skin cells. Cutan. Ocul. Toxicol. 29, 171–192.

McKim Jr., J.M., Keller 3rd, D.J., Gorski, J.R., 2012. An in vitro method for detecting chemical sensitization using human reconstructed skin models and its applicability to cosmetic, pharmaceutical, and medical device safety testing. Cutan. Ocul. Toxicol., 1–14 (Early online).

Miyazawa, M., Takashima, A., 2012. Development and validation of a new in vitro assay designed to measure contact allergen-triggered oxidative stress in dendritic cells. J. Dermatol. Sci. 68, 73–81.

Na, K., Kim, K.E., Park, S.T., Kim, T.Y., 2007. EC-SOD suppresses contact hypersensitivity in mouse skin by impairing Langerhans cell migration. J. Invest. Dermatol. 127, 1930–1937.

Naik, S.M., Cannon, G., Burbach, G.J., Singh, S.R., Swerlick, R.A., Wilcox, J.N., Ansel, J.C., Caughman, S.W., 1999. Human keratinocytes constitutively express interleukin-18 and secrete biologically active interleukin-18 after treatment with pro-inflammatory mediators and dinitrochlorobenzene. J. Invest. Dermatol. 113, 766–772.

Netzlaff, F., Lehr, C.M., Wertz, P.W., Schaefer, U.F., 2005. The human epidermis models EpiSkin, SkinEthic and EpiDerm: an evaluation of morphology and their suitability for testing phototoxicity, irritancy, corrosivity, and substance transport. Eur. J. Pharm. Biopharm. 60, 167–178.

Niwa, M., Nagai, K., Oike, H., Kobori, M., 2009. Evaluation of the skin irritation using a DNA microarray on a reconstructed human epidermal model. Biol. Pharm. Bull. 32, 203–208.

Nukada, Y., Ito, Y., Miyazawa, M., Sakaguchi, H., Nishiyama, N., 2011. The relationship between CD86 and CD54 protein expression and cytotoxicity following stimulation with contact allergen in THP-1 cells. J. Toxicol. Sci. 36, 313–324.

OECD, 2004. Guideline for Testing of Chemicals No. 431, In Vitro Skin Corrosion: Reconstructed Human Epidermis Test Method, adopted 13rd April.

OECD, 2010. Guideline for Testing of Chemicals No. 439, In Vitro Skin Irritation: Reconstructed Human Epidermis Test Method, adopted 22nd July.

Peiser, M., Tralau, T., Heidler, J., Api, A.M., Arts, J.H., Basketter, D.A., English, J., Diepgen, T.L., Fuhlbrigge, R.C., Gaspari, A.A., Johansen, J.D., Karlberg, A.T., Kimber, I., Lepoittevin, J.P., Liebsch, M., Maibach, H.I., Martin, S.F., Merk, H.F., Platzek, T., Rustemeyer, T., Schnuch, A., Vandebriel, R.J., White, I.R., Luch, A., 2012. Allergic contact dermatitis: epidemiology, molecular mechanisms, *in vitro* methods and regulatory aspects. Current knowledge assembled at an international workshop at BfR, Germany. Cell Mol. Life Sci. 69, 763–781.

Pollack, B.P., Sapkota, B., Haun, P.L., 2012. Activating transcription factor 3 (ATF3) expression is increased in erythema multiforme and is regulated by IFN-gamma in human keratinocytes. Exp. Dermatol. 19, e310–313.

Qiu, X.B., Shao, Y.M., Miao, S., Wang, L., 2006. The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. Cell Mol. Life Sci. 63, 2560–2570.

Ryan, C.A., Gildea, L.A., Hulette, B.C., Dearman, R.J., Kimber, I., Gerberick, G.F., 2004. Gene expression changes in peripheral blood-derived dendritic cells following exposure to a contact allergen. Toxicol. Lett. 150, 301–316.

Sasaki, Y., Aiba, S., 2007. Dendritic cells and contact dermatitis. Clin. Rev. Allergy Immunol. 33, 27–34.

Szameit, S., Vierlinger, K., Farmer, L., Tuschl, H., Noehammer, C., 2008. Microarray-based *in vitro* test system for the discrimination of contact allergens and irritants: identification of potential marker genes. Clin. Chem. 54, 525–533.

Szegezdi, E., Logue, S.E., Gorman, A.M., Samali, A., 2006. Mediators of endoplasmic reticulum stress-induced apoptosis. EMBO Rep. 7, 880–885.

Thompson, M.R., Xu, D., Williams, B.R., 2009. ATF3 transcription factor and its emerging roles in immunity and cancer. J. Mol. Med. (Berl.) 87, 1053–1060.

Toebak, M.J., Gibbs, S., Bruynzeel, D.P., Scheper, R.J., Rustemeyer, T., 2009. Dendritic cells: biology of the skin. Contact Dermatitis 60, 2–20.

Tsai, M.F., Wang, C.C., Chang, G.C., Chen, C.Y., Chen, H.Y., Cheng, C.L., Yang, Y.P., Wu, C.Y., Shih, F.Y., Liu, C.C., Lin, H.P., Jou, Y.S., Lin, S.C., Lin, C.W., Chen, W.J., Chan, W.K., Chen, J.J., Yang, P.C., 2006. A new tumor suppressor DnaJ-like heat shock protein, HLJ1, and survival of patients with non-small-cell lung carcinoma. J. Natl. Cancer Inst. 98, 825–838.

van Eijl, S., Zhu, Z., Cupitt, J., Gierula, M., Götz, C., Fritsche, E., Edwards, R.J., 2012. Elucidation of xenobiotic metabolism pathways in human skin and human skin models byproteomic profiling. PLoS One 7, e41721.

Vandebriel, R.J., Pennings, J.L., Baken, K.A., Pronk, T.E., Boorsma, A., Gottschalk, R., Van Loveren, H., 2010. Keratinocyte gene expression profiles discriminate sensitizing and irritating chemicals. Toxicol. Sci. 117, 81–89.

Vos, M.J., Hageman, J., Carra, S., Kampinga, H.H., 2008. Structural and functional diversities between members of the human HSPB, HSPH, HSPA, and DNAJ chaperone families. Biochemistry 47, 7001–7011.

Yang, H., Magilnick, N., Ou, X., Lu, S.C., 2005. Tumour necrosis factor alpha induces co-ordinated activation of rat GSH synthetic enzymes via nuclear factor kappaB and activator protein-1. Biochem. J. 391, 399–408.

Yusuf, N., Nasti, T.H., Huang, C.M., Huber, B.S., Jaleel, T., Lin, H.Y., Xu, H., Elmets, C.A., 2009. Heat shock proteins HSP27 and HSP70 are present in the skin and are important mediators of allergic contact hypersensitivity. J. Immunol. 182, 675–683.