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Evaluation of Potential Biomarkers for Thioacetamide-induced Hepatotoxicity using siRNA

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Abstract – In our previous publication we compared the gene expression profiles on hepatotoxicants exposure to assess the comparability between *in vivo* and *in vitro* test systems. We investigated global gene expression from both mouse liver and mouse hepatic cell line treated with thioacetamide (TAA) and identified several common genes. In this study, we selected genes to validate them as potential biomarkers for hepatotoxicity on the relevance of *in vitro* and *in vivo* system. Three up-regulated, aquaporin 8 (Aqp8), glutathione peroxidase 1 (Gpx1), succinate-CoA ligase, GDP-forming, alpha subunit (Suclg1) and two down-regulated, DnaJ (Hsp40) homolog subfamily C member 5 (Dnajc5) and tumor protein D52 (Tpd52) genes were tested for their effects *in vitro.* For characterization of gene function, short interfering RNA (siRNA) for each gene was synthesized and transfected in mouse hepatic cell line, BNL CL.2. Cell viability, mRNA expression level and morphological alterations were investigated. We confirmed siRNA transfection against selected five genes induced down-regulation of respective mRNA expression. siRNA transfection in general decreased cell viability in different degrees and induced morphological changes such as membrane thickening and alterations of intracellular structures. This suggests that these genes could be associated with TAA-induced toxicity. Furthermore, these genes may be used in the investigation of hepatotoxicity for better understanding of its mechanism.

Key words: Thioacetamide, Toxicogenomics, Hepatotoxicity, siRNA

INTRODUCTION

Evaluation of toxicity for a certain chemical is generally based on conventional toxicity tests, which presents usually several signs including changes of body weight, clinical value and morphology alterations, when toxic changes are evident. However, it is not effective when lesions are mild or moderate, and it takes more time to find out chronic toxicities.

As developments of many foods, drugs and chemicals with new technologies are more frequent, the demands for new technologies to evaluate them more quickly and accurately have increased not only for better safety evaluation but for cost-effectiveness. Among newly developed technologies, toxicogenomic approaches based on microarray allow to investigate expressions of thousands of genes affected simultaneously in biological experiments, and may serve as a valuable tool to evaluate chemicals.

Toxicogenomics combines transcript, protein and metabolite profiling with conventional toxicology (Waters and Fostel, 2004), and its approach should help not only to discover highly sensitive and predictive biomarkers for toxicity but also to understand molecular mechanism of toxicity (Pennie *et al.*, 2004). It seems that toxicogenomics will provide powerful tool that may show gene and protein changes earlier, even at treatment levels below the limits of detection of traditional measures of toxicity.

Our previous studies compared transcriptional responses between livers of animals and hepatocyte cell line after

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exposure to chemicals to determine how faithfully the *in vitro* model system reflects *in vivo* responses using microarray. Our results showed that it may be feasible to develop toxicogenomics biomarkers or profiles by comparing *in vivo* and *in vitro* genomic profiles specific to thioacetamide (TAA) for application to prediction of liver toxicity (Kang *et al.*, 2007).

Short interfering RNA (siRNA) is double-stranded RNA with 20-25 nucleotide, and siRNA was firstly discovered in plant as post-transcriptional gene silencing in 1999 (Hamilton and Baulcombe, 1999). Synthetic siRNA bound to single strand mRNA, with sequence specific manner (Dykxhoorn *et al.*, 2003) and induced RNAi in mammalian cells (Elbashir *et al.*, 2001). siRNA technique is effective way to reduce gene expression *in vitro*, and many experiments have been carried out to modulate gene expression with this approach both *in vitro* and *in vivo*.

In this study, we selected commonly altered down-regulated genes and up-regulated genes extracted from gene expression profiles from both mouse liver and mouse hepatic cell line treated with TAA, and we evaluated these genes as potential biomarkers for hepatotoxicity by transfection of respective siRNA.

MATERIALS AND METHODS

Materials

Murine embryonic normal hepatic cell line, BNL CL.2 cells (ATCC TIB-73) was purchased from American Type Culture Collection (ATCC, Manassas, VA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Invitrogen (Carlsbad, CA). CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay kit was obtained from Promega Co. (Madison, WI) while cytotoxicity detection kit was from Roche Molecular Biochemicals (Indianapolis, IN). TAA was obtained from Sigma (St. Louis, MO).

Cell line, cell culture

BNL CL.2 cells were cultured in DMEM medium supplemented with 100 units of penicillin-streptomycin/ml, 2 mM L-glutamine, and 10% FBS at 37°C in a 5% CO_2 atmosphere. TAA was dissolved in dimethyl sulfoxide (DMSO) and was freshly diluted in culture media for each experiment. Vehicle concentrations were less than 0.5% in all experiments.

siRNA synthesis and treatment

To validate potential biomarkers in TAA-induced hepatic toxicity, we selected several genes whose expression were regulated by TAA treatment based on our previous report (Kang *et al.*, 2007). Biological information for five selected gene are shown in Table I. siRNAs for these genes were prepared by Dharmacon Research (Lafayette, CO); three down-regulated genes such as aquaporin 8 (Aqp8), glutathione peroxidase 1 (Gpx1), succinate-CoA ligase, GDP-forming, alpha subunit (Suclg1), and two up-regulated genes such as DnaJ (Hsp40) homolog subfamily C member 5 (Dnajc5) and tumor protein D52 (Tpd52). We treated each siRNA into cells and examined the alteration of cell toxicity.

For setting of transfection condition, control (Zea mays) siRNA with carboxyfluorescein (Bioneer Co. Ltd, Korea)

Table I. Biological Information for selected genes

	Gene Title	Gene Symbol	mRNA Accession No	PANTHER Function	PANTHER Process
Down-regulated	aquaporin 8	Aqp8	NM_007474	Transporter	Transport; Homeostasis
	glutathione peroxidase 1	Gpx1	NM_008160	Oxidoreductase; Peroxidase	Detoxification: Immunity and defenes; Lipid, fatty acid and steroid metabolism; Antioxdation and free radical removal; Steroid metabolism; Immunity
	succinate-CoA ligase, GDP-forming, alpha	Suclg1	NM_019879	Synthase and synthetase	Tricarboxylic acid pathway; Carbohydrate metabolism
Up-regulated	DnaJ (Hsp40) homolog subfamily C, member 5	Dnajc5	XM_990872; NM_016775	Other chaperones; Membrane traffic protein; Chaperone	Synaptic transmission; Exocytosis; Neuronal activities; Neurotransmitter release; Protein folding; Intracellular protein traffic
	tumor protein D52	Tpd52	NM_001025261; NM_001025262; NM_001025263; NM_001025264; NM_009412	Molecular function unclassfied	Biological process unclassified

was transfected into cell using lipofectamine (RNAiMAX), and we carried out morphological examination using fluorescence microscope.

BNL CL2 cells ($2x10^4$ cells/ml; cell passage from 3 to 20) were set in 10% FBS-DMEM without antibiotics in 24 well-plate at 24 h before siRNA transfection. Solution A was prepared with mixing 50 μl DMEM (without antibiotics and FBS) with 1 μl of lipofectamine RNAiMAX (invitrogen) for 10 min, and solution B was prepared with mixing 50 μl DMEM (without antibiotics and FBS) with siRNA as final concentration as 5, 10, 20, 40 or 60 pmol for 10 min at RT. Solutions A and B (lipofectamin-siRNA complex) were mixed for 20 min and treated in cells during 48 h. Cell counts and microscopic examination were carried out at 6, 24 and 48 h. Data are the mean ± SD from three samples per group of three independent experiments.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR)

Total RNAs were extracted for gene expression analysis using the RNeasy Mini kit (Qiagene, Valencia, CA). The yield of RNA was determined by Bioanalyzer 2100 (Agilent Technology, CA), and stored at -80°C until use.

For cDNA synthesis TaqMan® Gold RT-PCR Kit (Applied Biosystmes, CA) was used according to manufacturer's guide. In brief, 2 μg of total RNA was mixed with 10 μl of 10X RT Buffer, 22 μl of 25 mM $MgCl_2$, 20 μl of deoxyNTPs mixture, 5 μl of random hexamers, 2 μl of RNase inhibitor, 2.5 μl of MultiScribe Reverse Transcriptase (50 U/μL) and incubate at 25°C for 10 min, at 37°C for 1 h, and at 95°C for 5 min and placed on ice for 10 min and stored at -20°C until use.

cDNAs were amplified against Aqp8, Gpx1, Suclg1, Dnajc5 or Tpd52 mRNA. PCR amplification was carried out according to the manufacturer's instruction (Applied Biosystems, CA). The PCR program cycles were set as follows: initial denaturing at 50°C for 20 min, 95°C for 10 min, followed by 40 cycles (95°C for 15 s, 60°C for 1 min).

Beta-actin mRNA was employed as an internal standard, and each gene expression was determined by RT-PCR and normalized against beta-actin mRNA levels. All PCR products were amplified in a linear cycle. Data are the mean ± SD from three samples per group of two independent experiments.

Morphological examination

BNL CL.2 cells were cultured in Chamber slide (Nunc, NY), and at 48 h after transfection, the cells were fixed in 0.5% glutaraldehyde (in PBS buffer, pH 7.4) for 15 min at 4°C,. The slides were stained with hematoxylin and were dipped into 1% acid alcohol, 0.2% ammonia water solution and eosin and morphological examination was carried out.

Statistical analysis

Statistical analyses were performed with the Tukey-Kramer method using the JMP program (SAS Institute, Cary, NC). For all comparisons, probability values less than 5% ($p<0.05$) were considered to be statistically significant.

RESULTS

Cell viability and mRNA level after siRNA transfection

Setting of transfection condition using control siRNA resulted in peak transfection occurred at 48 h after transfection (data not shown).

Aqp8 siRNA transfection slightly inhibited cell viability (Fig. 1A). However real-time RT-PCR analysis showed that Aqp8 siRNA transfection (5 pmol) effectively inhibited

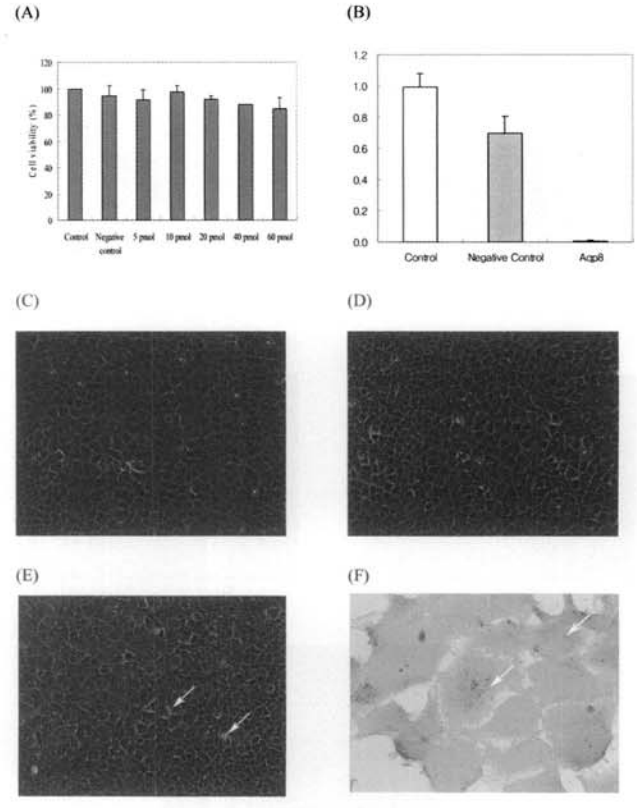

Fig. 1. Aquaporin 8 (Aqp8) siRNA transfection. (A) Dose-dependent treatment of siRNA and cell viability; (B) Aqp8 mRNA expression by siRNA transfection (5 pmol) measured by real-time RT-PCR; Morphological examination for control (C), negative control siRNA (D), Aqp8 siRNA (40 pmol) (E) and H&E staining (F). Note membrane thickening and alteration of intracellular structures (Arrow); Magnification, ×100 (C-E), ×1000 (F).

its mRNA expression at 48 h after treatment (Fig. 1B). Moreover morphological examination showed that compared to control (Fig. 1C) or negative control siRNA (Fig. 1D), there were membrane thickening and alteration of intracellular structures in samples of Aqp8 siRNA transfection (Fig. 1E), which also confirmed by H&E staining (Fig. 1F).

Gpx1 or Suclg1 siRNA transfection induced slight alterations of cell viability (Fig. 2A or Fig. 3A). However real-time RT-PCR analysis showed that Gpx1 or Suclg1 siRNA transfection (5 pmol) effectively inhibited its mRNA expression (Fig. 2B or Fig. 3B). Moreover morphological examination showed that compared to control (Fig. 2C or 3C) or negative control siRNA (Fig. 2D or 3D), there were cellular alterations existed in some cells treated with Gpx1 or Suclg1 siRNA transfection (Fig. 2E or Fig. 3E).

Dnajc5 or Tpd52 siRNA transfection inhibited cell viability by dose-dependent manner at 48 h. (Fig. 4A or Fig. 5A). Furthermore, real-time RT-PCR analysis showed that Dnajc5 or Tpd52 siRNA transfection (5 pmol) effectively inhibited its mRNA expression (Fig. 4B or Fig. 5B). And morphological examination showed that compared to control (Fig. 4C or 5C) or negative control siRNA (Fig. 4D or 5D), there were cellular alterations and cell death existed in some cells treated with Dnajc5 or Tpd52 siRNA transfection (Fig. 4E or Fig. 5E).

DISCUSSION

Our previous findings reported that there were significantly altered genes existed at 24 h more than at 6 h, representing toxic effects were enhanced at 24 h (Kang *et al.*, 2007). These data also showed gene expression profiles may provide useful methods for eliciting underlying molecular mechanism of drug susceptibility and of evaluating drug sensitivity *in vitro* correlated to *in vivo* about TAA toxicity.

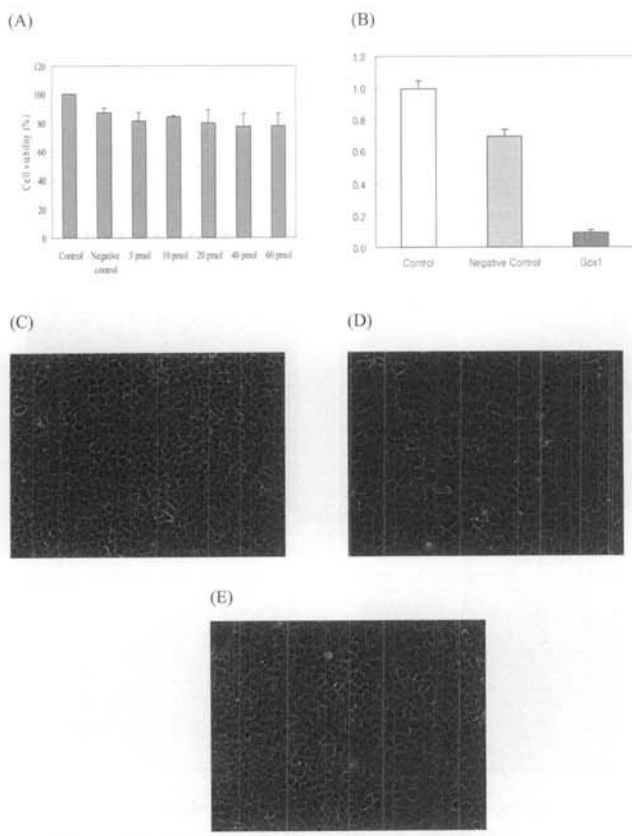

Fig. 2. Glutathione peroxidease 1 (Gpx1) siRNA transfection
(A) Dose-dependent treatment of siRNA and cell viability; (B) Gpx1 mRNA expression by siRNA transfection (5 pmol) measured by real-time RT-PCR; Morphological examination for control (C), negative control siRNA (D), Gpx1 siRNA (40 pmol) (E); Magnification, ×100 (C-E).

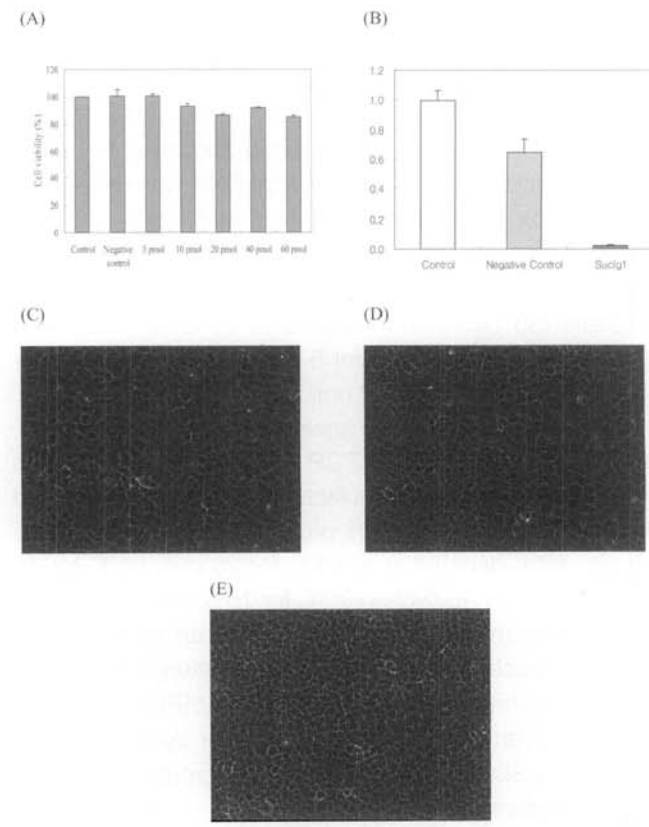

Fig. 3. Succinate-CoA ligase, GDP-forming, alpha subunit (Suclg1) siRNA transfection
(A) Dose-dependent treatment of siRNA and cell viability; (B) Suclg1 mRNA expression by siRNA transfection (5 pmol) measured by real-time RT-PCR; Morphological examination for control (C), negative control siRNA (D), Suclg1 siRNA (40 pmol) (E); Magnification, ×100 (C-E).

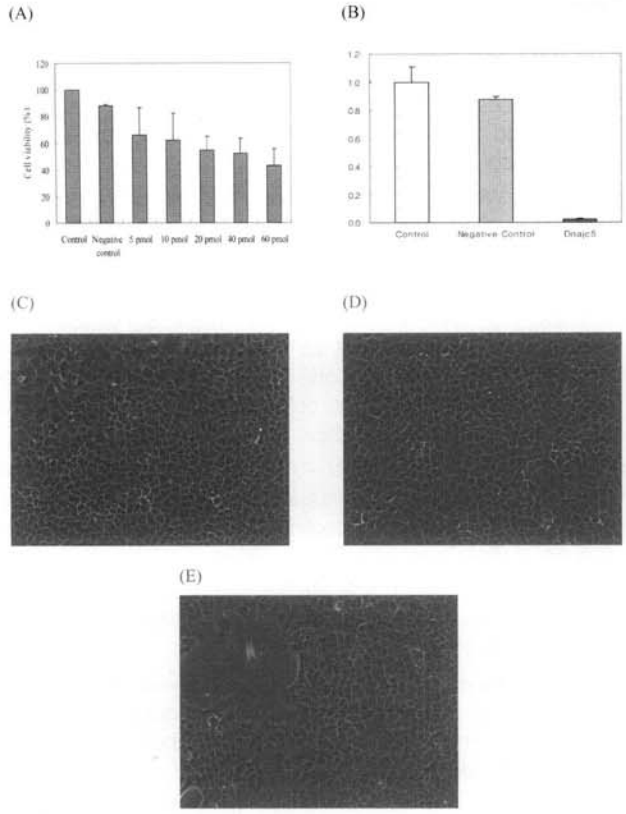

Fig. 4. DnaJ (Hsp40) homolog, subfamily C, member 5 (Dnajc5) siRNA transfection
(A) Dose-dependent treatment of siRNA and cell viability; (B) Dnajc5 mRNA expression by siRNA transfection (5 pmol) measured by real-time RT-PCR; Morphological examination for control (C), negative control siRNA (D), Dnajc5 siRNA (40 pmol) (E); Magnification, ×100 (C-E).

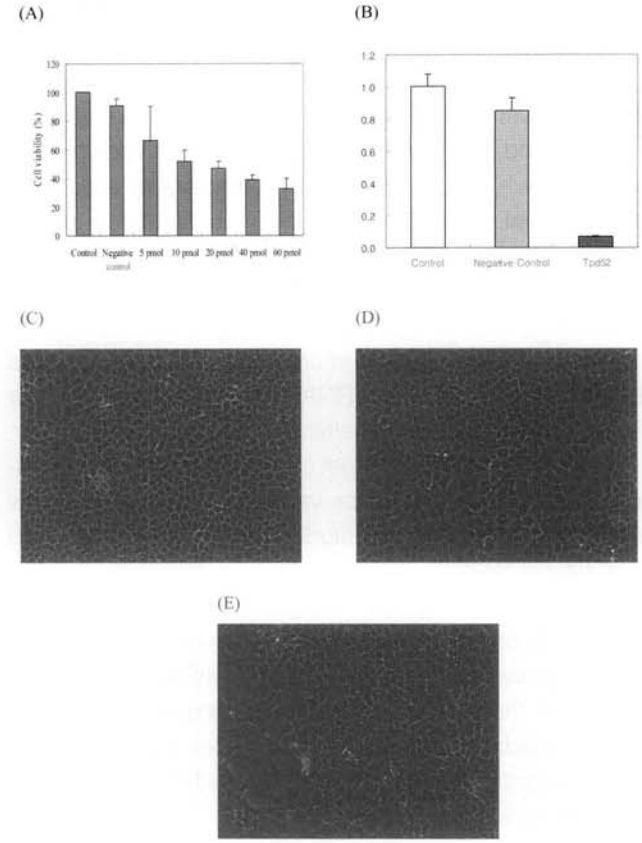

Fig. 5. Tumor protein D52 (Tpd52) siRNA transfection
(A) Dose-dependent treatment of siRNA and cell viability; (B) Tpd52 mRNA expression by siRNA transfection (5 pmol) measured by real-time RT-PCR; Morphological examination for control (C), negative control siRNA (D), Tpd52 siRNA (40 pmol) (E); Magnification, ×100 (C-E).

We picked up commonly up- or down-regulated genes between *in vivo* and *in vitro* at 24 h, and evaluated the potential markers for liver toxicity using siRNA. To set siRNA transfection condition using Zea mays siRNA, we found that the transfection was occurred from 6 h to 48 h, with high frequency at 48 h after transfection, and siRNA transfection for target genes showed that cell death was occurred from 6 to 48 h, with high frequency at 48 h. It was impossible to extent the transfection time owing to cell confluence.

siRNA tranfection for Aqp8, Gpx1 or Suclg1 showed that cell viabilities were not generally altered. However, gene expression levels were down-regulated at 48 h, and furthermore morphological alterations were shown. Especially, Aqu8 siRNA transfection induced cell membrane thickening and alteration of intracellular structures.

It was reported that Aqp8 was essential component of cell membrane and was involved in water transport (Ma *et al.*, 1997), and inhibition of Aqp8 expression by siRNA significantly decreased osmotic water permeability in isolated colonocytes (Laforenza *et al.*, 2005). So, it seems siRNA transfection for Aqp8 may induce alterations of water transport and cellular osmolarity in hepatic cells.

It was reported that Gpx1 was in glutathione peroxidase family and was involved in steroid metabolism, stress response and detoxicification, and alteration or abnormality of Gpx1 expression was associated with chronic diseases (Lei *et al.*, 2007). TAA toxicity was associated with increase of oxidative stress and activation of hepatic stellate cells (Kang *et al.*, 2005; Li *et al.*, 2002; Muller *et al.*, 1988; Porter *et al.*, 1979). As glutathione peroxidase mRNA expression was greatly down-regulated in liver of wild-type mice at 24 and 48 h after TAA treatment (Kang *et al.*, 2008), siRNA transfection for Gpx1 could inhibit detoxicification process and enhance oxidative stress in hepatic cells, resulting in hepatic cell alterations. As Suclg1 is involved in TCA cycle

(Lambeth *et al.*, 2004), siRNA transfection for Scu1g1 may induce the alteration of cell metabolism.

siRNA tranfection for Dnajc5 or Tpd52 showed that cell viabilities were also down-regulated at dose-dependent manner, and gene expression levels were down-regulated at 48 h, and furthermore morphological alterations were evident. Dnajc5 is involved in heat shock protein binding (Ohtsuka and Hata, 2000), and Tpd52 has protein heterdimerization activity and protein homodimerization activity, and it is thought that it may be involved in cellular transformation, tumorigenesis and metastasis (Lewis *et al.*, 2007). So, siRNA transfection for Dnajc5 or Tpd52 may induce cellular alterations and cell death.

siRNA transfection for target genes is useful tool to investigate their mechanism. For validation of these potential biomarkers *in vivo*, further studies will be warranted using short hairpin RNA.

Taken together, siRNA transfection of these genes induced alterations of cell viability and morphology as well as an effective inhibition of respective mRNA, and it suggests that these genes could be associated with TAA-induced toxicity. Furthermore, these genes may be used in the investigation of hepatotoxicity for better understanding of its mechanism.

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