Identification of pro-apoptotic genes as toxicity-predictive biomarkers using hES-derived hepatocyte-like cells

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Identification of pro-apoptotic genes as toxicity-predictive biomarkers using hES-derived hepatocyte-like cells

Directed by Professor Yun-Han Lee

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ABSTRACT

Identification of pro-apoptotic genes as toxicity-predictive biomarkers using hES-derived hepatocyte-like cells

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Toxicogenomics is a new emerging field which combines toxicology with genomics to understand the toxicity and efficacy of drugs in drug development. The altered gene expression patterns induced by reference toxic compounds show the list of potential toxic molecules involved in cytotoxic signal pathway causing adverse drug effects. One of the most important issues for the application of potential toxicity-predictive biomarkers discovered by toxicogenomics is to establish human biology-based toxicity test because there are some limitations to extrapolate between animal models and human, such as

differences of metabolic enzymes among species. Drug-induced liver injury (DILI) is a phenomenon of adverse drug activity. It is important to identify potential toxicity-predictive biomarkers associated hepatotoxicity.

In this study, microarray data from hES-derived hepatocyte-like cells treated with known hepatotoxic drugs, namely APAP, diclofenac, and thioacetamide, was analyzed by Ingenuity Pathway Analysis (IPA). Based on molecular networks predicted by IPA, we choose to up-regulated pro-apoptotic proteins; ATF3 and GADD34 as target genes with cytotoxic properties. We confirmed that mRNA and protein levels of ATF3 and GADD34 were increased after treating with the three hepatotoxic drugs, corresponding with IPA results. We further identified the ER stress pathway consisting of ATF3 and GADD34, ultimately culminating apoptosis. Collectively, these data suggested that hepatotoxic drugs induce pro-apoptotic proteins which are involved in ER stress-mediated apoptosis. Such toxic proteins could be a useful toxicity-predictive biomarker for evaluating a potential hepatotoxicant triggering ER stress.

Key words: toxicogenomics, toxicity-predictive biomarkers, hepatotoxicity, hES-derived hepatocyte-like cells, ER stress-mediated apoptosis

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I. INTRODUCTION

Drug-induced liver injury (DILI) is a phenomenon of adverse drug activity, ultimately leading to a liver failure. DILI could basically be divided into two categories. One is an acute hepatocellular injury, which is irreversible cellular damage, such as hepatocellular apoptosis and necrosis; the other is chronic hepatocellular injury, including hepatic fibrosis and cirrhosis.

DILI has actually been a great part of the causes of failure in drug development and withdrawal of an approved drug.[1] Although there are numerous efforts to

assess hepatotoxicity in preclinical drug development, the pre-clinical in vitro models of non-human origin systems such as mice and rats could not be shown to drug toxicity correctly because of the species differences.[2,3] There are also some limitations for using the human hepatocytes and liver tissues, which are isolating and culturing of primary human hepatocytes as well as liver specimen that displays individual differences.[4] To overcome these issues, hepatocytes differentiated from human embryonic stem cells (hESCs) have emerged as an optimal cell type to evaluate toxic effects of candidate drugs in vitro.

Toxicogenomics is a new field which combines toxicology with genomics. This technology provides a comprehensive understanding of drug's activity, especially an aspect of molecular mechanism, compared with classical toxicology that enables to show histopathological effects and organ weight changes in the last time (endpoint).[5,6] Genome-wide expression profiles obtained by toxicogenomics provide transcriptome changes between control groups and candidate drugs treated groups. Altered gene expression patterns are analyzed and then the results can predict plausible cellular events to induce adverse drug activity such as toxicity pathway, stress signaling or cell death pathway.[7] Several assays that evaluate drug toxicity have currently developed. One of them is the ToxTracker Assay which enables to detect visually by using

green fluorescent protein (GFP) reporter systems. For example, Srnx1-GFP reporter system is activated under oxidative stress conditions. If a certain candidate drug induces oxidative stress, ToxTracker Assay shows Srnx1-GFP fluorescence as a marker gene of oxidative stress signaling.[8] This method is a very useful tool because detection of a toxic molecule can be performed easily by comparison of fluorescence intensity. Fluorescence indirectly represents toxic properties of a compound in molecular level. For establishing advanced in vitro toxicity assay to detect protein associated with cellular stress signaling, it is necessary to identify a specific molecule with the potentially toxic properties as a toxicity-predictive marker for cellular damage.

ER is an abundant organelle in cell types with secretory characteristics such as hepatocyte and plays a crucial role in protein folding and modification. When unfolded protein burden is beyond the capacity of ER, the unfolded protein response (UPR) is activated to maintain the function of ER and cellular homeostasis.[9] Due to prolonged ER stress, however, cells fail to perform protective and adaptive response and then cells may initiate a mechanism of programmed cell death known as apoptosis.[10] It has been reported that ER stress is one of the major cellular toxicity pathways triggered by hepatotoxicant.[11-13]

Here we studied the pro-apoptotic proteins induced after exposure of three

different hepatotoxic drugs; APAP (acetaminophen), diclofenac and thioacetamide using human embryonic stem cell-derived hepatocyte-like cells, which more accurately reflect biological systems than any other non-human in vitro model. We performed microarray and differentially expressed genes were analyzed by using Ingenuity Pathway Analysis (IPA) software. We sought to focus on up-regulated pro-apoptotic proteins in highly ranked molecular networks predicted by IPA and finally choose to ATF3 and GADD34 as target genes with cytotoxic properties. We validated that the mRNA and protein levels of ATF3 and GADD34 increased after treating hepatotoxic drugs. We also confirmed ER stress pathway consisting of ATF3 and GADD34, ultimately culminating apoptosis. Our results suggest that these pro-apoptotic genes induced by hepatotoxic drugs could become a useful toxicity-predictive biomarker, especially showing cytotoxic properties triggering ER stress, for drug discovery.

II. MATERIALS AND METHODS

1. Cell culture

The hES-derived hepatocyte-like cells used in result1 were purchased from Cellartis (Cellartis Inc., Gothenburg, Sweden). The hES-derived hepatocyte-like cells used in result2 and result3 were kindly provided by Prof. Jong-Hoon Kim.[14] Cells were incubated in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) (Gibco, Carlsbad, CA, USA), supplemented with 1% of penicillin/streptomycin solution and 1% Insulin Transferrin Selenium (ITS) plus oncostatin M (OSM, 10ng/ml) and dexamethasone (DEX, 10^{-6}mol/L) at 37°C with 5% CO_2.

2. Drug treatment

The hES-derived hepatocyte-like cells were individually treated with the IC50 values of Acetaminophen (APAP; Sigma-Aldrich cat#A7085-100G), Diclofenac sodium salt (Sigma-Aldrich cat#D6899-10G), Thioacetamide (Sigma-Aldrich 163678-25G). All drugs are diluted in culture media.

3. Cell viability assay

Cell viability assay was measured using the Cell Counting Kit-8 (CCK-8)

(Dojindo Molecular Technologies, Inc., Tokyo, Japan) as recommended by the manufacturer. hES-derived hepatocyte-like cells were plated in triplicate into a 96-well plates and were treated with hepatotoxic drugs for 24hours at 37℃ in a humidified, 5% CO_2 atmosphere. CCK-8 solution was added in a well to 10% of the total volume. The colored products (formazan) were detected by measuring the absorbance at 450nm using a VersaMax microplate reader (Molecular Device, Sunnyvale, CA, USA).

4. Gene expression analysis

hES-derived hepatocyte-like cells were treated with the individual IC50 values of APAP, diclofenac and thioacetamide. After 6hr and 12hr, total RNA was isolated and then applied to the microarray analysis using GeneChip Human Genome U133 plus 2.0 array (Affymetrix Inc., Santa Clara, CA, USA). The biological function, toxicological functions and network analysis were performed using Ingenuity Pathway Analysis (QIAGEN / Ingenuity Systems, Inc. Redwood City, CA) by Prof. Seo, Daekwan (Head of Bioinformatics Core, Seoul National University).

5. RT-PCR analysis

The Purification of total RNA was performed by Trizol (Ambion Inc., Texas, USA). Chloroform (100ul/500ul trizol) was added in the sample treated with Trizol. After vortexing for 10 sec, samples were incubated on room temperature for 5min. Next, samples were centrifuged at 12,000g at 4℃ for 15min to separate three layer in the tube. The transparent upper layer containing total RNA was collected in a new tube, and then 250ul of isopropanol was added in each sample, followed by softly inverting the tube. Afterward, the sample was centrifuged at 12,000g at 4℃ for 10min to obtain the pellet. The pallet was washed in 75% ethanol diluted in Nuclease-Free Water (NFW) (Ambion Inc., Texas, USA). The washed pellet was centrifuged at 12,000g at 4℃ for 5min. The pallet was finally dried at room temperature for approximately 5 min.

The evaluation of quality and quantity of the RNA was performed by NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA) which automatically calculates the OD 260/280 ratio.

Complimentary DNA (cDNA) was synthesized by using PrimeScript^{TM} 1st strand cDNA Synthesis Kit (Takara Biotech, Kusatsu, Shiga, Japan) according to the manufacturer's instructions.

The cDNAs were obtained and amplified by using PrimeSTAR HS DNA

Polymerase (Takara Biotech, Kusatsu, Shiga, Japan) in PCR conditions as follows: cycles of denaturation at 98℃ for 10sec, annealing at 55℃ for 15sec, and elongation at 72℃ for 1min/kb. The primers used for PCR were GAPDH, 5'-GGG AGC CAA AAG GGT CAT CAT CTC-3' (sense) and 5'-CCA TGC CAG TGA GCT TCC CGT TC-3' (antisense); CPS1, 5'-CAA GGA GCC ATT GTT TGG AA-3' (sense) and 5'-CCA ATG GAC AGA CCT CCT GA-3' (antisense); AAT, 5'-ACT GTC AAC TTC GGG GAC AC-3' (sense) and 5'-CCC CAT TGC TGA AGA CCT TA-3'(antisense); and albumin, 5'-CTT CCT GGG CAT GTT TTT GT-3' (sense) and 5'-GGT TCA GGA CCA CGG ATA GA-3' (antisense).

The PCR products were separated by using 1.2% agarose gel electrophoresis and visually detected by MiniBis Pro Gel documentation system (DNR Bio-Imaging Systems, Jerusalem, Israel) after staining with Loading Star (Dyne Bio, Seoul, Korea).

6. Real-time PCR analysis

Total RNA was isolated as mentioned above and was synthesized to cDNA by using using PrimeScript^{TM} 1st strand cDNA Synthesis Kit (Takara Biotech, Kusatsu, Shiga, Japan) according to the manufacturer's instructions.

Next, each cDNA sample was loaded in the LightCycler 480 96well plate (Roche, Basel, Switzerland) and then added mixture containing SYBR Premix Ex Taq^{TM} (Tli RNaseH Plus) (Takara Biotech, Kusatsu, Shiga, Japan) and the following primers: GAPDH, 5'-GGG AGC CAA AAG GGT CAT CAT CTC-3' (sense) and 5'-CCA TGC CAG TGA GCT TCC CGT TC-3' (antisense); GADD45B, 5'-GTG TAC GAG TCG GCC AAG TT-3' (sense) and 5'-TGT CAC AGC AGA AGG ACT GG-3' (antisense); BCL2L11, 5´-TGG CAA AGC AAC CTT CTG ATG-3' (sense) and 5´-GCA GGC TGC AAT TGT CTA CCT-3' (antisense); DDIT-3, 5'-CCT CAC TCT CCA GAT TCC A-3' (sense) and 5'-AGC CGT TCA TTC TCT TCA G-3' (antisense); ACVR1C, 5'- TAT GAT GTG ACC GCC TCT GG-3' (sense) and 5'−TCT GCC TCA CGA AAC CAA GA-3' (antisense); PPP1R15A, 5'- CCC AGA AAC CCC TAC TCA TGA TC-3' (sense) and 5'- GCC CAG ACA GCC AGG AAA T-3' (antisense); ATF-3, 5'-GGA GTG CCT GCA GAA AGA GT-3' (sense) and 5'- CCA TTC TGA GCC CGG ACA AT-3' (antisense).

PCR conditions for SYBR Green-based real-time PCR were set in following protocol: pre incubation 1cycle, 95°C for 600sec; 3 step amplification 45 cycle, 95°C for 10sec, 60°C for 10sec and 72°C for 10sec; melting 1 cycle, 95°C for 10sec, 65°C for 60sec and 97°C for 1sec.

The relative quantification of mRNA level was automatically analyzed by LightCycler 96 software version 1.1, comparing with the Ct (threshold cycle) values of each target gene.

7. Western blot analysis

The cells were lysed in RIPA buffer (Thermo Scientific, Rockford, IL, USA) that consists of 0.01% of a protease and phosphatase inhibitor cocktail (Thermo Scientific, Rockford, IL, USA). The amount of protein was quantified by using the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Equal amounts of proteins were separated by SDS-PAGE on a 12% gel and transferred to polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). The membrane was then blocked with 5% skim milk/Tris – buffered saline containg Tween 20 (TBS-T) for 1hr at room temperature. After washing the membranes with TBS-T, the membrane was incubated with primary antibodies against GADD34 (sc-8327, Santa Cruz Biotechnology, CA, USA), GRP78 (sc-13968, Santa Cruz Biotechnology, CA, USA), ATF-3 (sc-188, Santa Cruz Biotechnology, CA, USA) for overnight at 4°C. The membranes was washed three times in TBS-T for 10min each and then was incubated with horseradish peroxidase (HRP) goat

anti-mouse IgG (Santa Cruz) and HRP goat anti-rabbit IgG (Santa Cruz) horseradish peroxidase-conjugated secondary antibodies for 2hr at room temperature. The membrane was washed three times in TBS-T for 10min each. Finally, immunoreactive bands were visually detected by using LAS-3000 Imager (Fujifilm Corporation, Tokyo, Japan). The antibody of β-actin was used as a loading control to assess the equal loading volume.

8. Statistical analysis

Results are expressed as the mean ± SE (Standard Error) from triplicate independent experiments. In comparison of two groups, statistical significance was calculated by Student's t-test. *p<0.05, **p<0.01.

III. RESULTS

1. Hepatotoxic Drugs alter gene expression profiles, including especially apoptosis signaling-related genes.

To investigate global gene expression profiles altered by hepatotoxic drugs, we exposed hES-derived hepatocyte like cells to commonly known three hepatotoxic drugs; APAP, diclofenac and thioacetamide. APAP is reported as capable of inducing hepatocyte apoptosis and necrosis[15-17] and diclofenac is known to trigger hepatocyte apoptosis, autoimmune hepatitis.[18-21] Thioacetamide is known to induce hepatocyte apoptosis, necrosis, fibrosis and cirrhosis.[22-24] These three hepatotoxic drugs commonly induce cell death via apoptosis.

Firstly, we identified the IC50 values of three hepatotoxic drugs by performing cell viability assay because we speculated that the concentration of IC 50 values exhibit the gene expression changes including both survival and death related genes. APAP showed approximately 50% survival of cells at concentration from 50mM to 100mM and diclofenac also had 50% survival of cells at concentration around 1000mM. However, survival of the cells treated with thioacetamide significantly decreased on a rage from 30mM to 100mM but gradually

increased after then. This result may be due to dissolve beyond the solubility limit. The IC50 value of thiacetamide for hES-derived hepatocyte-like cells was calculated to 77mM (Figure 1). We used the chosen concentration for further the analysis of microarray.

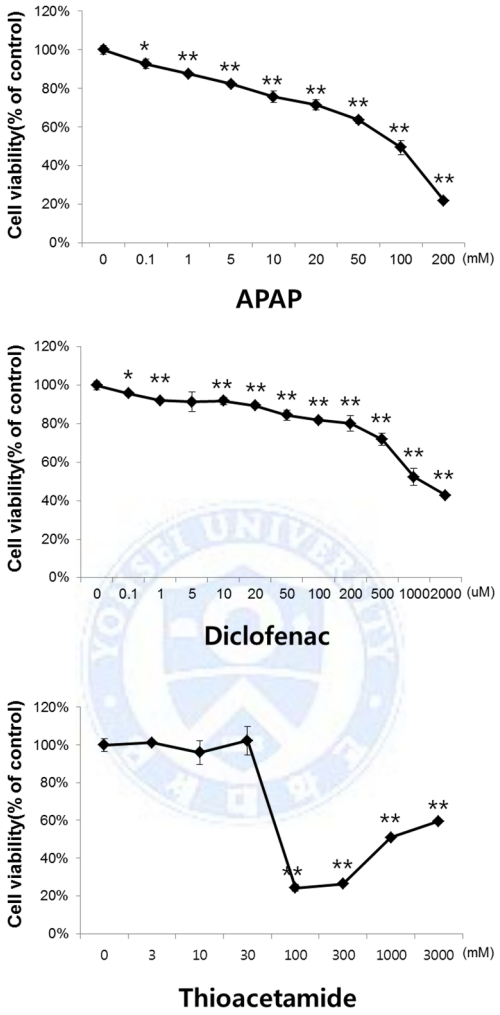
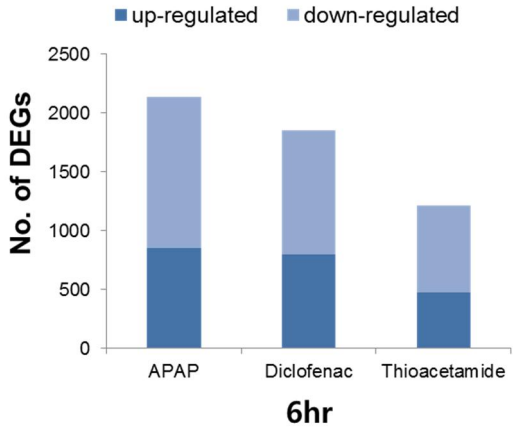
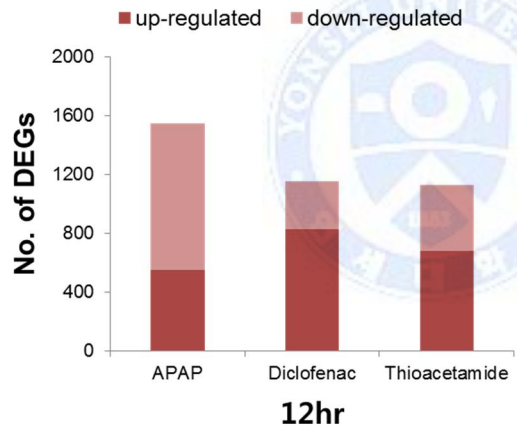


Figure 1. Cell viability of hES-derived hepatocyte-like cells after 24hr treatment with APAP, diclofenac and thioacetamide. To calculate IC50 values of individual drugs in hES-derived hepatocyte-like cells, the cell were treated with various concentrations of each drug. After 24hr, cell viability assay was performed using CCK-8.

Next, to identify changes of gene expression pattern triggered by hepatotoxic drugs and biological mechanisms induced by the toxic drugs, we performed microarray analysis and then microarray datasets were analyzed by using Ingenuity Pathway Analysis (IPA) software. APAP induced the largest gene expression changes in the IC 50 value. The number of genes with two or more fold changes is 2,133 in treatment of APAP for 6hr and 1,548 in treatment of APAP for 12hr. Whereas thioacetamide showed minimal effects on gene expression changes (Figure 2).



6hr	Up	Down
APAP	850	1283
Diclo	800	1051
Thio	475	736



12hr	Up	Down
APAP	552	996
Diclo	827	328
Thio	680	451

Figure 2. The number of upregulated genes or downregulated genes following treatment with APAP, diclofenac and thiocetamide. Differentially Expressed Genes (DEGs) were determined by microarray analysis dataset after individually exposure to IC50 values of hepatotoxic drugs for 6hr and 12hr.

Also, we identified the number of genes perturbed by commonly two or three hepatotoxic drugs, particularly in both 6hr and 12hr commonly. The total number of up-regulated genes by commonly two or three drugs was 107. While that of down-regulated genes was 191, especially among them, 187 was in overlapping portion of both APAP and thioacetamide. (Figure 3) The genes which were commonly expressed in two or three drugs data sets (107 genes up- and 191 down-regulated genes) were used in IPA for further analysis.

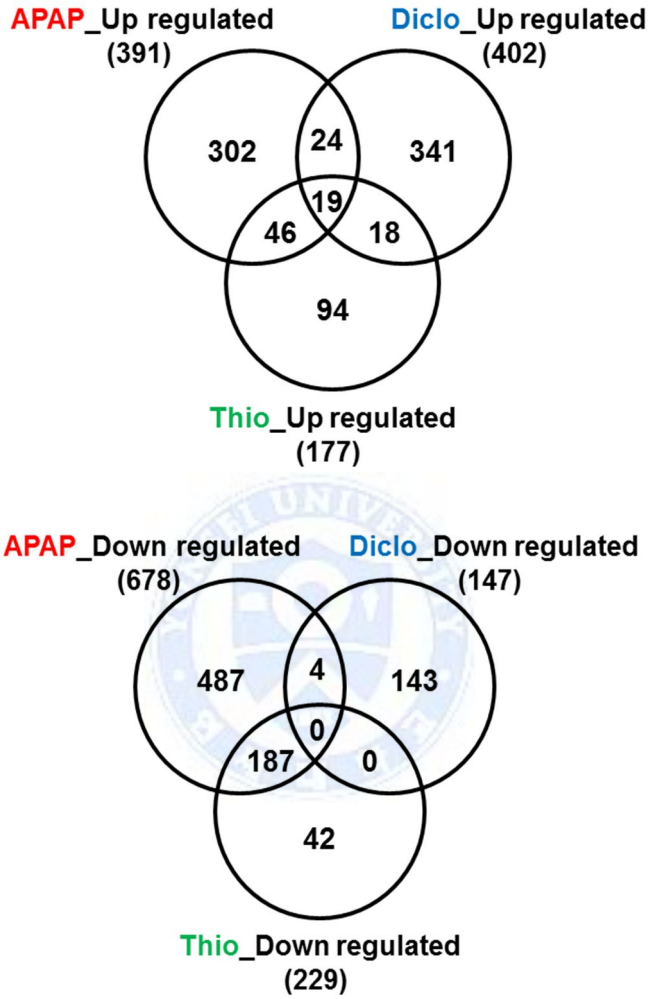


Figure 3. The number of differentially expressed genes altered in common both 6hr and 12hr. The number of genes induced by two or three hepatotoxic drugs in common both 6hr and 12hr was shown in overlapping portion of venn-diagrams.

The IPA results showed that the list of Tox function associated with hepatotoxicity and determined their respective p-value. We identified that Liver Necrosis/Cell Death, Liver Proliferation, Liver Inflammation/Hepatitis, Hepatocellular Peroxisome Proliferation, Liver Hyperplasia/Hyperproliferation were ranked by statistical significance. In the list of Tox function, Liver Necrosis/Cell Death came out to be the most significant cytotoxic pathway with a p-value of 7.50E-05 – 2.88E-02 (Figure 4).

Top Tox Functions

Hepatotoxicity

Name	p-value
Liver Necrosis/Cell Death	7.50E-05 - 2.88E-02
Liver Proliferation	1.45E-04 - 4.90E-01
Liver Inflammation/Hepatitis	1.13E-03 - 3.06E-01
Hepatocellular Peroxisome Proliferation	1.45E-02 - 1.45E-02
Liver Hyperplasia/Hyperproliferation	1.89E-02 - 5.59E-01

Figure 4. The list of top five tox functions associated with hepatotoxicity for up-regulated genes(107) and down-regulated genes(191) affected by two or three hepatotoxic drugs in common. The list of Top Toxicity Functions related to hepatotoxicity ranked by their respective p-value determining the probability.

The IPA constructed the plausible biologic networks for the 107 genes up- and 191 down-regulated genes in overlapping portion of two-or three hepatotoxic drugs data sets (Figure 5). The green color represented downregulation of gene expression and red color represented upregulation of gene expression.

To determine which genes among up-regulated genes in the highly ranked top five networks are related to apoptotic effects, we sought to explore the apoptotic functions of each up-regulated gene by searching for literature sources. As a results of, we identified there were six genes associated with positive regulation of apoptosis, namely GADD45B, BCL2L11, DDIT3, ACVR1C, PPP1R15A (GADD34) and ATF3 (Table 1, Figure 6).

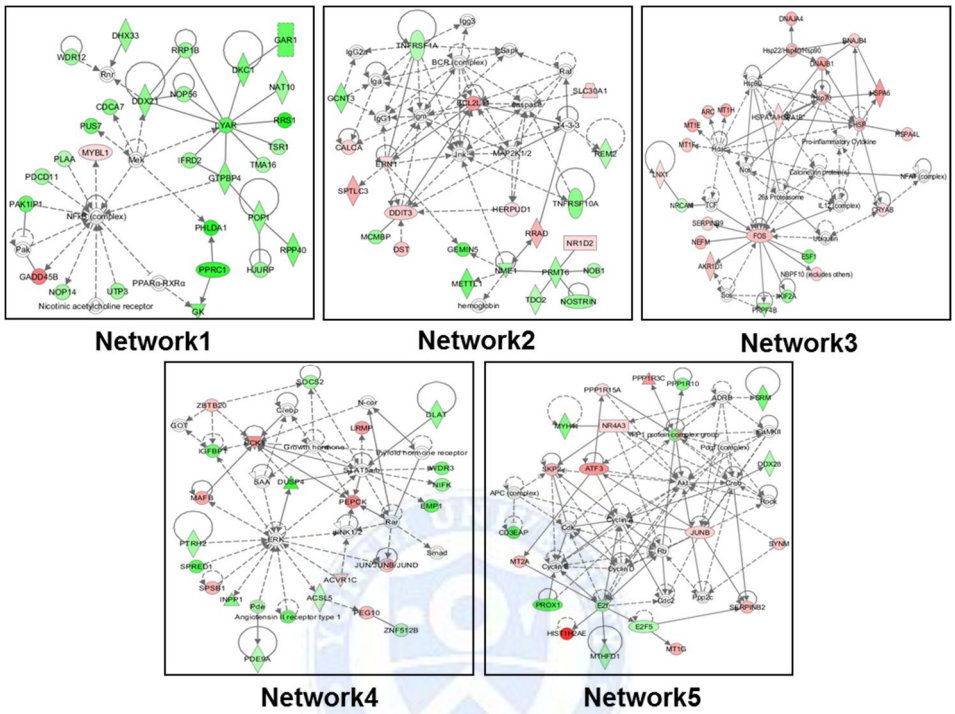
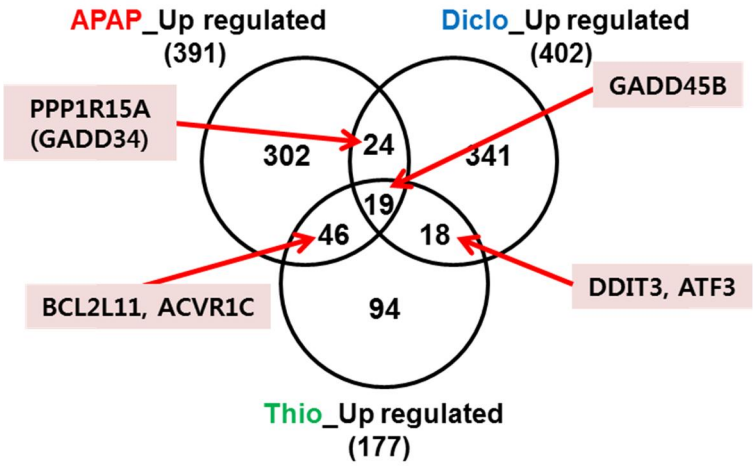


Figure 5. The highly ranked top five biologic networks for up-regulated genes(107) and down-regulated genes(191) affected by two or three hepatotoxic drugs in common. The biologic networks obtained from IPA showed plausible functional relationships of various proteins. The green and red colors indicated down- and up-regulated genes respectively. Also, gray color represented the degree of regulated gene did not show significance.

Table 1. The list of up-regulated genes in the highly ranked top five networks for up-regulated genes (107) and down-regulated genes (191) affected by two or three hepatotoxic drugs in common

Network #	Gene name	Pass drugs	Pro-apoptotic function
Network 1	MYBL1	2	
	GADD45B	3	√
Network 2	BCL2L11	2	√
	SLC30A1	2	
	CALCA	2	
	ERN1	2	
	SPTLC3	2	
	DDIT3	2	√
	DST	2	
	HERPUD1	2	
	RRAD	3	
	NR1D2	2	
Network 3	DNAJA4	2	
	DNAJB4	2	
	Hsp22/Hsp40/Hsp90	1	
	DNAJB1	3	
	Hsp70		
	HSPA6	3	
	HSPA4L	2	
	MT1H	3	
	MT1E	3	
	MT1F	3	
	ARC	2	

	LNX1	2	
	SERPINB9	3	
	NEFM	3	
	AKR1D1		
	FOS	3	
	NBPF10	2	
	CRYAB	3	
Network 4	ZBTB20	2	
	PCK1	2	
	LRMP	2	
	MAFB	2	
	PEPCK		
	JUN/JUNB/JUND	2	
	ACVR1C	2	√
	SPSB1	2	
	PEG10	2	
Network 5	PPP1R15A	2	√
	PPP1R3C	2	
	NR4A3	2	
	SKP2		
	ATF3	2	√
	JUNB	2	
	SYNM	2	
	MT2A	3	
	HIST1H2AE	2	
	MT1G	3	
	SERPINB2	2	



Gene Function	Gene Name
Positive regulation of apoptosis	GADD45B
	BCL2L11
	DDIT3
	ACVR1C
	PPP1R15A
	ATF3

Figure 6. The pro-apoptotic genes among up-regulated genes in the highly ranked top five networks. The positions of six genes associated with apoptosis pathway were displayed in venn-diagrams stated in Figure 3.

2. The mRNA level of ATF3 and GADD34 are mainly induced by hepatotoxic drugs

We evaluated hepatospecific markers such as CPS1, AAT, albumin before using hES-derived hepatocyte-like cells for further experiments. The expression of liver specific markers in hES-derived hepatocyte-like cells that show fully differentiated cells mature hepatic cells was assessed by RT-PCR, comparing with HepG2 cells as positive controls. The hES-derived hepatocyte-like cells expressed AAT and albumin, similar to the HepG2 cells. Also, CPS1 was more markedly expressed in hES-derived hepatocyte-like cells (Figure 7).

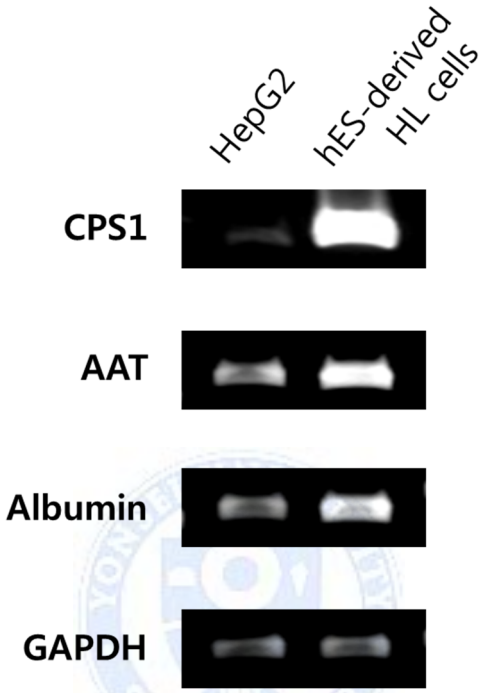


Figure 7. Validation of hepatospecific markers on hES-derived hepatocyte-like cells. In comparison with HepG2 cells as a positive control, hepatospecific markers such as CPS1, AAT and albumin in ES-derived hepatocyte-like cells were detected by RT-PCR.

To corroborate whether the actual expression patterns of six apoptotic genes (GADD45B, BCL2L11, DDIT3, ACVR1C, PPP1R15A and ATF3) could correspond with IPA data, we identified the expression level of mRNA of six apoptotic genes in hES-derived hepatocyte-like cells treated with three hepatotoxic drugs. Diclofenac markedly induced the mRNA expression of ATF3, GADD34 and DDIT3 in both 6hr and 12hr. The increase of GADD45B was triggered by APAP and thioacetamide, respectively, but not diclofenac. ATF3 and GADD34 were commonly induced in hES-derived hepatocyte-like cells individually treated with three hepatotoxic drugs in both 6hr and 12hr, comparing with untreated cells as control. However, the mRNA expression of ALK7 and BCL2L11 were induced only by thioacetamide (Figure 7).

These data indicated that the three hepatotoxic drugs induced a variety of apoptotic genes in hES-derived hepatocyte like cells and also commonly triggered induction of ATF3 and GADD34. Thus, we selected ATF3 and GADD34 as target genes with pro-apoptotic properties that increased generally under hepatotoxic conditions.

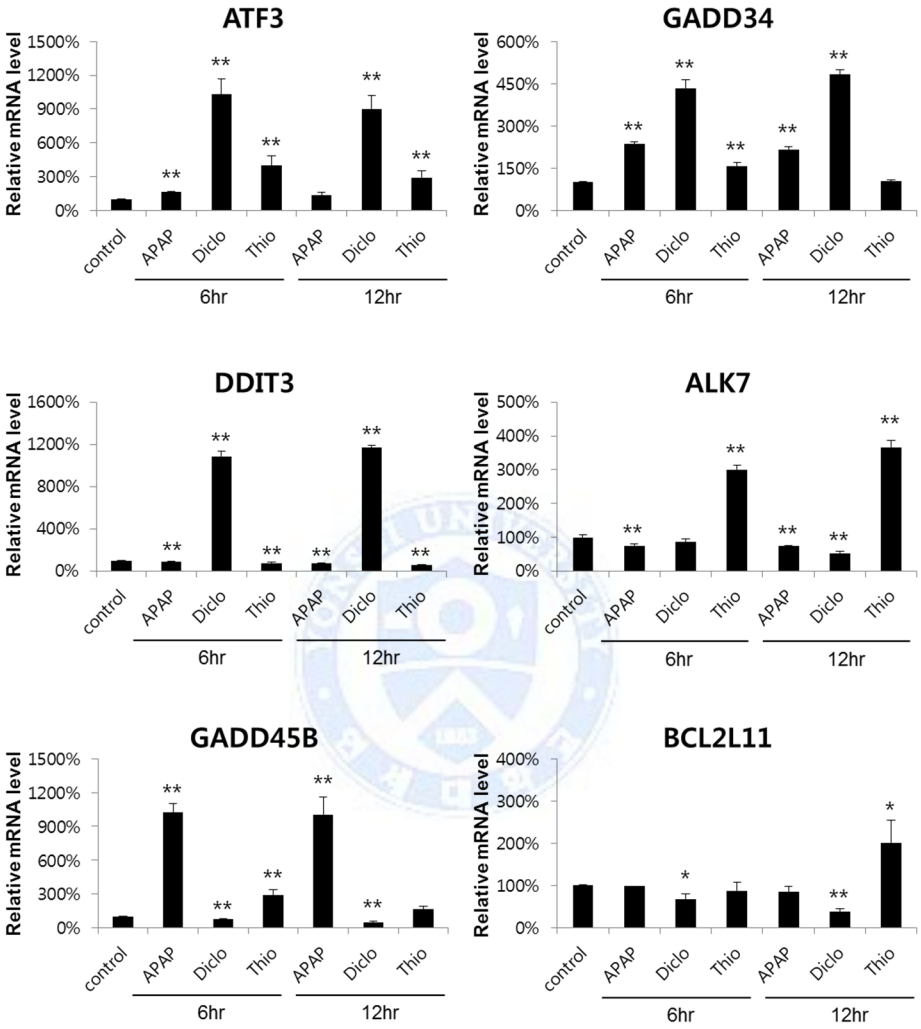


Figure 8. The mRNA levels of pro-apoptotic genes selected by IPA results. To verify the IPA results, real-time PCR analysis was performed on ATF3, GADD34, DDIT3, ALK7, GADD45B and BCL2L11.

3. The protein level of ATF3 and GADD34 were induced by hepatotoxic drugs, followed by ER stress-mediated apoptosis.

Next, we wanted to identify that hepatotoxic drugs induce the protein levels of ATF3 and GADD34 as well as mRNA levels validated by qRT-PCR. We chose diclofenac representatively because diclofenac significantly induced the mRNA levels of ATF3 and GADD34. Thus, we anticipated that the diclofenac could apparently show the changes of protein levels. As shown Figure 8, the protein level of ATF3 was obviously induced in hES-derived hepatocyte-like cells treated with diclofenac for 36hr. We also identified increasing the protein level of GADD34. ATF3 and GADD34 are involved in ER stress pathway. The protein level of GRP78 known for an ER stress marker was induced by treating with diclofenac. Moreover, the expression of pro-apoptotic protein Bax increased after treatment with diclofenac (Figure 8).

These results demonstrated that hepatotoxic drugs induced the ATF3 and GADD34, followed by ER stress-mediated apoptotic pathway.

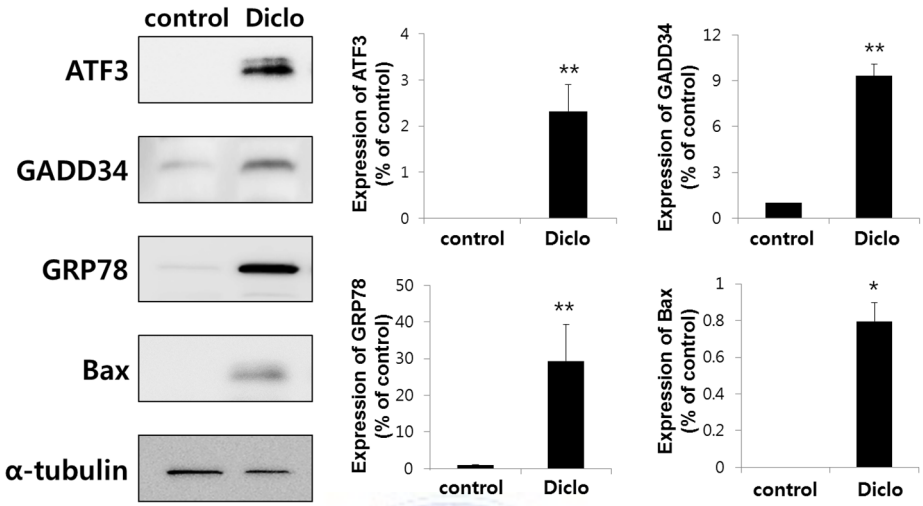


Figure 9. The protein levels of ATF3, GADD34 and ER stress-mediated apoptotic signal in response to diclofenac. The protein levels of ATF3, GADD34, GRP78 and Bax was determined 36hr after treatment with diclofenac. The level of α-tubulin protein was used as loading control of western blot.

IV. DISCUSSIONS

Hepatotoxic drugs are the most major cause of the drug-induced liver injury and also result in to be withdrawn of approved drugs.[25] Even though in vitro and animal models in preclinical stages have been used to evaluate toxicity, hepatotoxicity is revealed in the late stage of drug development such as clinical trials because of limitation of extrapolation from animal such as differences of metabolic enzymes among species.[26] In response to this issue, human biology-based toxicity test using cell lines derived from human embryonic stem cell is required.

In this study, we exposed hES-derived hepatocyte-like cells to known hepatotoxic drugs, namely APAP, diclofenac and thioacetamide. These three drugs have been reported to induce hepatocyte cell death via apoptosis.

We investigated the gene expression patterns altered in the conditions of drug-induced liver injury based on genomics dataset that were obtained by microarray. We used 107 genes up- and 191 down-regulated genes in overlapping portion of venn-diagrams which represented the number of differentially expressed genes by three hepatotoxic drugs for IPA. As a result, IPA constructed the plausible biologic molecular networks. We sought to explore the pro-apoptotic genes among up-regulated genes in the highly ranked

top five networks. We identify six pro-apoptotic genes, namely GADD45B, BCL2L11, DDIT3, ACVR1C, PPP1R15A (GADD34) and ATF3. Then, we verified whether IPA data would correspond with actual up-regulated genes by qRT-PCR and finally chose to focus on the ATF3 and GADD34 that comprise eIF2α signaling of ER stress pathway.[27] We also identified that hepatotoxicity drugs induced the protein levels of ATF3 and GADD34 and also induced ER stress marker, GRP78 by western blot assay. Moreover, pro-apoptotic protein, Bax increased by treating with hepatotoxic drugs.

ATF3 known for stress response gene is a member of ATF/CREB family transcription factors.[28] Various studies currently showed that ATF3 is stress-inducible gene, which is rarely detected in normal conditions but induced by genotoxic agents, apoptosis stimulus, and ischemic as well as hypoxic conditions. Moreover, especially in liver, the mRNA expression level of ATF3 increased in hepatocyte treated some chemicals such as carbon tetrachloride, alcohol, acetaminophen (APAP), and also induced in hepatic ischemia.[29] However, ATF3 also has been considered to be either pro-apoptotic or anti-apoptotic factor.[30] This may be due to differentially interpreting of experimental results, in other words, ATF3 is induced to manage cellular damage or is an output resulted from cellular damage. In any case, ATF3 is

certainly induced in cellular stress conditions. The induction of ATF3 is an early response to various stimuli. In the left atrium of hearts, the level of ATF3 mRNA was early induced after treating with angiotensin II as stimulus.[31] Also, in case of dopaminergic neuronal cell line, MN9D, ATF3 was robustly increased by treating with toxin treatment as a role of early stress-induced transcription factor.[17] Our data demonstrated that the protein levels of ATF3 was not detected in normal hES-derived hepatocyte-like cells but strongly induced by hepatotoxic drugs. Though the target genes of ATF3 are not well understood, recent report indicated that ATF3 contribute to induction of GADD34 expression.[32]

Upon ER stress, GADD34 dephosphorylates eIF2α, reversing translational inhibition. Reversing of translational inhibition facilitates accumulation of unfolded proteins load and translation of pro-apoptotic mRNA.

Even though GADD34 and ATF3 is not hepatocyte specific ER stress marker, these proteins play an important role for ER stress in multiple tissues. Thus, GADD34 and ATF3 are used as ER stress markers in many studies. Interestingly, our results showed that DDIT3, one of the ER stress markers, was not induced in treatment with hepatotoxic drugs.

ER stress exists in all cell types. However, especially, in hepatocytes with

secretory properties, ER contents are about 50% of all cellular organelle and ER stress is reported as an important toxic pathway in many liver diseases including nonalcoholic fatty liver disease, hyperhomocysteinemia and cholestatic liver disease. Thus, ER stress seems to play an crutial role in hepatotoxic events.[34]

The advance of toxicogenomics has an impact on field of toxicology regarding to more comprehensively understanding activity of adverse drugs in the aspect of molecular mechanism. Also, it is anticipates that an approach of toxicogenomics enables to reduce the cost and time of drug development as comparison with classical toxicity test that would take a long time to preclinical animal test and require so many animal models.[33] Thus, a number of research groups have already been engaged in discovery and identification of toxicity-predictive biomarkers or biomarker signatures to predict toxic properties of candidate drugs in early stage of drug development pipelines. These toxicity-predictive biomarkers also provide more valuable information about the molecular mechanism of cellular toxicity. For example, if the expression level of genes involved in DNA damage repair increased by certain compounds, that compounds may be considered as genotoxic agents. Recently, Giel et al. developed the novel toxicity assay, called ToxTracker Assay, which is GFP reporter systems selectively activated in potential genotoxic compounds.

These assay systems have advantages on providing mechanistic information with high sensitivity.

Out data suggests that several pro-apoptotic genes were upregulated by treating with hepatotoxic drugs and especially ATF3 and GADD34 are induced in response to ER stress-mediated apoptotic conditions triggered by hepatotoxic drugs. Therefore, establishing of a detectable vector that consist of pro-apoptotic genes activated in the conditions of ER stress triggered by hepatotoxicants such as ATF3 and GADD34 provides prediction tools of hepatotoxic compounds. These vector systems applied for a variety of cell lines derived from human embryonic stem cell could be useful tools to evaluate drug toxicity, displaying certain stress-signaling pathways.

V. CONCLUSION

These data suggest that pro-apoptotic proteins involved in ER stress-mediated apoptosis are induced in hES-derived hepatocyte-like cells treated with hepatotoxic drugs. Such toxic proteins could be useful toxicity-predictive biomarkers displaying certain cytotoxic signaling pathway for establishing toxicity assay system on drug discovery.

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ABSTRACT (IN KOREAN)

인간배아줄기세포유래간세포에서 독성예측 생체지표로 활용하기 위한 세포사멸 유전자들의 기능 확인

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약물의 부작용으로 발생하는 약인성 간손상(Drug-induced liver injury)은 승인된 약물이 시장에서 퇴출되는 주요 원인이다. 따라서 잠재적인 간 독성 예측 생체지표를 발굴하여 신약 개발 과정에서 사전에 간 독성을 정확하게 평가하는 것은 매우 중요하다.

독성유전체학은 기존의 독성학에 유전체학을 접목한 분야로써, 신약 개발 과정에서 신약 후보 물질의 독성과 효능을 평가하는데 활용할 수 있는 분야로 떠오르고 있다. 이미 알려진 독성 물질을 처리함으로써 달라지는 유전자 발현 패턴은 약물 부작용을 일으키는 세포 독성 신호 전달 과정에 관련되어 있는 잠재적인 독성 단백질이 무엇인지

보여 준다.

이러한 독성 단백질을 독성예측 생체지표로 발굴하여 독성 평가 시스템 구축에 적용하는데 있어서 중요한 점은 동물 모델 기반 시스템을 생체 시스템에 정확히 적용하기에는 한계가 있으므로, 인체 생리학적 특성이 반영된 인체 세포 기반에서 독성을 평가할 수 있는 시스템을 구축해야 한다는 것이다.

따라서 본 연구에서는, 인간배아줄기세포로부터 유래된 간세포를 이용하여 간 독성을 일으키는 것으로 알려진 APAP, diclofenac, thioacetamide 약물을 처리한 후, 달라지는 유전자 발현 패턴을 파악하기 위해 microarray를 시행하였다. 그런 뒤, 두 가지 이상의 약물에서 공통적으로 변화하는 유전자들을 가지고 Ingenuity Pathway Analysis (IPA) 소프트웨어를 이용하여 분석하였다. IPA 결과로 예측된 상위 5가지 분자 네트워크 중에서, 발현이 증가된 유전자들 중 세포 사멸과 관련된 유전자들을 문헌적으로 조사하여 6가지 유전자들(GADD45B, BCL2L11, DDIT3, ACVR1C, GADD34, ATF3)로 범위를 좁혔고, 그 중에서도 GADD34와 ATF3의 메신저 RNA 수준이 실제로 증가됨을 real-time PCR 결과를 바탕으로 확인하였다. GADD34와 ATF3는 세포 내 소포체 스트레스 신호 전달 경로를 이루는 단백질들이며, 과도하게 오랫동안 지속되는 소포체 스트레스는 세포사멸로

이어지게 된다. GADD34와 ATF3의 단백질 증가와 소포체 스트레스 마커인 GRP78, 세포사멸 마커인 Bax의 단백질 발현 증가를 western blot을 통해 확인하였다.

따라서 이러한 결과들은 간 독성 약물이 소포체 스트레스 통한 세포 사멸을 일으킨다는 것을 보여주며, 이러한 신호 전달 과정에 나타나는 독성 단백질들은 간 독성 약물을 평가할 수 있는 독성평가 시스템을 구축하는데 있어서 소포체 스트레스를 보여 줄 수 있는 세포독성 생체지표로 유용하게 활용될 수 있을 것이라 기대한다.

핵심되는 말: 독성유전체학, 독성예측 생체지표, 간 독성,
인간배아줄기세포 유래 간세포, 소포체 스트레스로 인한 세포사멸