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Gene Expression Analysis of Anticancer Drug Induced Hepatotoxicity Using cDNA Microarray

Gyoung-Jae Lee[1], Yang-Suk Kim[2], Jin-Wook Jung[3], Seung-Yong Hwang[3], Joon-Suk Park[4], Kyung-Sun Kang[4], Yong-Soon Lee[4], Man-Suk Chon[1], Kum-Jin Chon[1], Jong-Soo Kang[1], Dong-Hyean Kim[5] & Young-Keun Park[6]

[1]Research Institute. Shin-Won Scientific Co., Ltd., #603 Kumbong Technovalley II 93-5, Dangjeong-dong, Gunpo-si, Gyeonggi-do 435-81, Korea
[2]Bioinformatics Unit, ISTECH Inc., #704 Hyundai Town Vill, 848-1 Janghang, Ilsan, Goyang, Gyeonggi-do 411-380, Korea
[3]Department of Biochemistry and Molecular Biology, Hanyang University & Genocheck Co., Ltd, Sa 1-dong, Sangnok-gu, Ansan, Gyeonggi-do 426-791, Korea
[4]Department of Veterinary Public Health, College of Veterinary Medicine, Seoul National University, San 56-1, Shilm-dong, Kwanak-gu, Seoul 151-742, Korea
[5]KIST, 39-1 Hawolgok-dong, Seongbuk-gu, Seoul 136-791, Korea
[6]Department of Biotechnology and Genetic Engineering Korea University, Anam-dong, Seongbuk-gu, Seoul 136-701, Korea
Correspondence and requests for materials should be addressed to Y.K. Park (ykpark@korea.ac.kr)

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Abstract

Tamoxifen (TAM), a non-steroidal antiestrogen anticancer drug and chemopreventive agent for breast cancer, have caused cholestasis in liver. The potent hepatocarcinogenicity of this drug has been reported. Methotrexate (MTX) is dihydrofolate reductase inhibitor which interfaces with the synthesis for urine nucleotide and dTMP. And it may cause atrophy, necrosis and steatosis in liver. These two anticancer drug have well-known hepatotoxicity. So, in this study we compare the gene expression pattern of antitumor agent TAM and MTX, using the cDNA microarray. We have used 4.8 K cDNA microarray to identify hepatotoxicity-related genes in 5-week-old male Sprague-Dawley (SD) rats. Confirm the pattern of gene expression, we have used Real time PCR for targeted gene. In the case of MTX, Protease related gene (Ctse, Ctsk) and Protein kinase (Pctk 1) have shown specific expression pattern. And in the case of TAM, apoptosis related gene (Pdcd 8) and signal transduction related gene (kdr) have significantly up regulated during treatment time. Gene related with growth factor, lipid synthesis, chemokins were significantly changed. From the result of this study, the information about influence of TAM and MTX to hepatoxicity will provide.

Keywords: Toxicogenomics, Antitumor agent, Hepatotoxicity, Microarray, Hepatocarcinogenesis

One of the main reasons for the failure of drug candidate during the development process is unpredictable toxicity or insufficient efficacy at *in vivo* animal model. Therefore numerous approaches are to investigate the relationship between chemical exposure and toxic effect. That is to say, the toxic effect produced by drug exposure is result of complex interaction from one or multiple cellular pathways, which can be detected by the analysis of various gene expressions. The field of toxicogenomics, using the microarrays and bioinformatics, has the potential to advance our understanding of genes are involved in responses of chemical exposure[1-3].

Hepototoxicity is one of most common adverse events resulting in drug withdraws from the market. This finding raises the question of how effective preclinical and clinical testing are in recognizing hepatotoxicity. The ultimate goal is to use DNA microarray as a screening tool in order to determine the hepatototoxicity and side effects of new chemical entities[4].

In order to confirm the concept of toxicogenomics, several kind of cDNA chip was developed. For these purposes, we were developed the 4.8 K cDNA chip (Table 1) and that chip was used to study for hepatotoxicity of antitumor agent[5].

Tamoxifen (TAM), a non-steroidal antiestrogen anticancer drug and chemopreventive agent for breast cancer, have caused cholestasis[6]. And potent hepato-

Table 1. Gene content list of the 4.8 K cDNA chip.

Related gene	Number	Related gene	Number
Apoptosis	6	Intracellular	406
Cancer	29	Membrane	18
Cell communication	8	Nuclear acid binding	130
Cell cycle regulation	27	Signal transducer	93
Chaperon	8	Signal transduction	151
Enzyme	684	Structural protein	39
Extracellular	40	Transporter	119
Immunity protein	13	The others (including ESTs)	3229

Table 2. Body weight exchange.

Group	Drug	Body weight (g)					
		−1 Day	1 Day	4 Day	7 Day	11 Day	14 Day
13	Control (Corn oil)	117.8	133.9	164.1	166.8		
14	TAM (20 mg/kg)	119.4	134.7	149.0	149.6		
15	TAM (68 mg/kg)	118.1	133.3	142.6	141.1		
16	Control (Saline)	116.8	137.1	164.0	168.6		
17	MTX (0.25 mg/kg)	119.1	138.2	164.8	172.9		
18	MTX (1 mg/kg)	121.0	140.2	169.1	169.4		
19	Control (Corn oil)	116.2	133.3	161.2	195.8	217.0	220.7
20	TAM (20 mg/kg)	114.7	127.8	143.4	162.4	170.0	181.8
21	TAM (68 mg/kg)	116.1	130.4	143.9	161.2	177.1	179.8
22	Contro (Saline)	114.1	132.0	160.2	193.8	219.6	218.9
23	MTX (0.25 mg/kg)	114.8	133.2	161.0	197.2	224.6	229.1
24	MTX (1 mg/kg)	114.7	132.9	159.9	194.6	210.9	204.6

carcinogenicity of this drug has been reported[7]. So, some researchers have been applied to toxicogenomic approach to the hepatocarcinogenesis in female rat[8]. Methotrexate (MTX) is dihydrofolate reductase inhibitor which interfaces with the synthesis for urine nucleotide and dTMP. And it may cause atrophy, necrosis and steatosis[9]. So, many researcher also have been used this drug for toxicogenomic study[10,11].

In this study we tested these two antitumor agents, which have different hepatotoxicity. Using our own developed cDNA chip we confirmed the hypothesis that the relationship of gene expression profile and their hepatotoxicity are closely related.

Clinical Observation and Histopathology

All animal were exposed the two anticancer drug (MTX and TAM) for 1, 3, 7 and 14 days. All animals were observed daily for clinical sign and checked the body weight at twice a week.

No mortality was observed and physical observations were generally not significant during this study.

In the case of body weight, the body weights of TAM treatment group were decreased significantly according to the treatment concentration ($p < 0.05$) (Table 2).

All animals were conducted the blood chemistry analysis, hematology, organ weighing and histopathological analysis. And no significant alteration were observed of all treatment animals except relative organ weight. The 14 day treatment groups of TAM and MTX have showed significant increasing in relative liver weight statistically (Table 3).

Gene Expression Changes Induced by Treatment with the Drug

In order to determine gene expression changes associated with chemical treatment, liver total RNA was collected after 1, 3, 7 and 14 days of each chemical treatment. Competitive hybridizations of fluorescently labeled cDNA derived from control vs. treated liver samples were used to measure relative abundance of each mRNA on the 4.8 K cDNA chip. The data were normalized using internal standard.

Table 3. Relative Liver weight.

Group	Drug	Liver weight (Relative, g)
13	Control (Corn oil)	4.0
14	TAM (20 mg/kg)	4.0
15	TAM (68 mg/kg)	3.9
16	Control (Saline)	3.9
17	MTX (0.25 mg/kg)	3.7
18	MTX (1 mg/kg)	3.7
19	Control (Corn oil)	4.3
20	TAM (20 mg/kg)	5.0
21	TAM (68 mg/kg)	5.1
22	Control (Saline)	3.9
23	MTX (0.25 mg/kg)	4.1
24	MTX (1 mg/kg)	5.2

From the analysis data, the identified genes, which were changed their expression profiling by TAM and MTX treatment, were described at table 4 and 5. In the case of MTX, the 24 genes have specific expression patterns. Gene expression profiling of MTX high dose treatment group showed that 10 genes were up regulated, e.g, Hadha (long fatty acid oxidation related gene), DNAi (inhibitor of DNA binding 3), cal III (calmodulin III). And in these group 14 genes were down regulated, e.g, Cts E (cathepsin E), Cts K (cathepsin K), TFIIA, Pctk 1 (Cdk relate kinase family), Lyp III (Lipophospholipase II). And MTX low dose group showed that 9 genes were down regulated, e.g, EST-cal (similar to S50193 Ca^{2+}/calmodulin-dependent protein kinase). In the case of TAM high dose treatment group, 10 genes were up regulated, e.g, kdr (VEGF 2 receptor), pdcd 8 (apoptosis-inducing fac-

Table 4. Gene expression profiling of MTX.

1) MTX-1 mg/kg/day

Gene symbol	Description	Fold change
	Up regulated	
HAP 1	huntingtin-associated protein 1	66.79
Best 5	Best5 protein	48.29
Cyto	cytolysin	23.07
EST-jc	ESTs, Weakly similar to JC4296 ring finger protein-fruit fly (Drosophila melanogaster) [D. melanogaster]	13.25
Hadha	hydroxyacyl-Coenzyme A dehydrogenase/3-ketoacyl-Coenzyme A hiolase/enoyl-Coenzyme A hydratase (trifunctional protein), alpha subunit	6.58
EST-col	ESTs, Highly similar to serologically defined colon cancer antigen 28; phosphatidylcholine transfer protein-like [Mus musculus] [M. musculus]	4.83
DNAi	Inhibitor of DNA binding 3, dominant negative helix-loop-helix protein	4.56
Cal III	Calmodulin III	4.37
Cbp/p300	Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2	3.44
Eph A1	ephrin A1	1.42
	Down regulated	
EST-PAI	ESTs, Weakly similar to S19896 plasminogen activator inhibitor 2 type A-rat [R. norvegicus]	−1.10
EST-U	EST Ubiquitin-conjugating enzyme E2H (predicted)	−3.18
EST2	ESTs	−3.55
SC6	sodium channel, voltage-gated, type 6, alpha polypeptide	−6.27
EST-btd	ESTs, Moderately similar to biotinidase [Mus musculus] [M. musculus]	−8.02
LypIII	lysophospholipase II	−8.31
Cts K	cathepsin K	−9.00
EST-Imp	ESTs, Highly similar to IMB3_HUMAN Importin beta-3 subunit (Karyopherin beta-3 subunit) (Ran-binding protein 5) [H. sapiens]	−16.31
EST1	ESTs	−18.06
EST-Pai-1	ESTs, Weakly similar to plasminogen activator inhibitor 2 type A [Rattus norvegicus] [R. norvegicus]	−23.55
Cts E	Cathepsin E	−24.44
TFIIA	Rattus norvegicus TFIIA small subunit mRNA, complete cds	−31.76
Pctk 1	PCTAIRE-1 protein kinase, alternatively spliced	−62.14
EST-eEF	ESTs, Weakly similar to S21055 translation elongation factor eEF-1 alpha chain -rat [R. norvegicus]	−64.93

2) MTX-0.25 mg/kg/day

Gene symbol	Description	Fold change
	Down regulated	
EST-SPK 24	ESTs Similar to Serine/threonine protein kinase 24	−1.73
AATF	Rattus norvegicus mRNA for AATF protein (transcription factor)	−1.15
2-Glp	alpha-2-glycoprotein 1, zinc	−1.24
Lat-3	calcium-independent alpha-latrotoxin receptor homolog 3	−3.10
EST-URF	EST, Moderately similar to 0806162J protein URF4 [Mus musculus] [M. musculus]	−3.14
EST-Cal	ESTs, Weakly similar to S50193 Ca^{2+}/calmodulin-dependent protein kinase EC 2.7.1.123) I-rat [R. norvegicus]	−3.62
Glp 4	glutathione peroxidase 4	−3.80
EST-RER	ESTs, Highly similar to similar to S. cerevisiae RER1 [Homo sapiens] [H. sapiens]	−7.09
EST-UCy	ESTs, Highly similar to UCR2_RAT Ubiquinol-cytochrome C reductase complex core protein 2, mitochondrial precursor (Complex III subunit II) [R. norvegicus]	−48.79

Table 5. Gene expression profiling of TAM.

1) TAM-68 mg/kg/day

Gene symbol	Description	Fold change
	Up regulated	
kdr	FLK1 kinase insert domain receptor (VEGF receptor 2)	18.59
Pdcd 8	programmed cell death 8 (apoptosis-inducing factor)	16.33
ago61	ESTs, Highly similar to hypothetical protein FLJ14566 [Homo sapiens] [H. sapiens]	10.65
EST-PTP	ESTs	9.37
Fib	fibrillin-2	9.03
Discs	Drosophila discs-large tumor suppressor homologue (synapse associated protein)	8.05
ESTs	ESTs	6.20
PRKC	PRKC, apoptosis, WT1, regulator	3.87
ESTs	ESTs	3.40
ESTs	ESTs, Weakly similar to ZF37_RAT Zinc finger protein 37 (Zfp-37) [R. norvegicus]	1.73
ESTs	ESTs, Weakly similar to ZF37_RAT Zinc finger protein 37 (Zfp-37) [R. norvegicus]	1.73
	Down regulated	
EST-CM	ESTs cellular morphogenesis during differentiation (GO:0000904); embryonic development (sensu Mammalia) (GO:0001701); protein binding (GO:0005515); protein binding (GO:0005515); receptor mediated endocytosis (GO:0006898); development (GO:0007275); neurogenesis (GO:0007399); excretion (GO:0007588); cell proliferation (GO:0008283); negative regulation of cell growth (GO:0030308); No_GO	−2.08
Pro 2A	Rat senescence marker protein 2A gene, exons 1 and 2	−6.09
Cyp	cytochrome P450, 2b19	−11.29
EST-SY21	ESTs, Moderately similar to SY21_MOUSE Small inducible cytokine A21 precursor (CCL21) (Beta chemokine exodus-2) (6Ckine) (Thymus-derived chemotactic agent 4) (TCA4) [M. musculus]	−44.52

2) TAM-20 mg/kg/day

Gene symbol	Description	Fold change
	Up regulated	
kdr	FLK1 kinase insert domain receptor (VEGF receptor 2)	16.59
GP	unknown Glu-Pro dipeptide repeat protein	11.24
Nrt	neuronatin	9.48
Gld	Glutamate dehydrogenase	7.56
BMP6	Bone morphogenetic protein 6	5.85
intgrin	integrin-associated protein	3.57
aducine	Adducin 3, gamma	3.43
Pro	proteasome (prosome, macropain) subunit, alpha type 4	2.31
	Down regulated	
EST-cc	ESTs, Highly similar to serologically defined colon cancer antigen 28; phosphatidylcholine transfer protein-like [Mus musculus] [M. musculus]	−1.96
EST-BMP	ESTs, Moderately similar to BMP1_MOUSE Bone morphogenetic protein 1 precursor (BMP-1) (Procollagen C-proteinase) (PCP) (Mammalian tolloid protein) (mTld) [M. musculus]	−2.25
EST-Mat	ESTs, Highly similar to A57235 RING finger protein MAT1-mouse [M. musculus]	−3.78

tor), PRKC (apoptosis related gene) and 4 genes were down regulated, e.g, EST-SY21 (similar to SY21_MOUSE Small inducible cytokine A21). And TAM low dose group showed that 8 genes were up regulated, e.g, kdr, BMP-6 and 3 genes were down regulated., e.g, EST-cc (imilar to serologically defined colon cancer antigen 28).

Table 6. Sequence of primer using real time PCR.

Gene	Primer Sequence	Orientation	Gene name
Ctsk	AACGACCAAGTTCACAGCTCAC	sense	cathepsin K
	CTCCATGCCTGTAACACTCCAG	antisense	
Ctse	AGCTGCGTGGCTATTTATCACA	sense	Cathepsin E
	TCAGACCAAACAAAGCATGAGA	antisense	
Pctk1	ACTGGTTGGGCACTACTCCCT	sense	PCTAIRE-1 protein kinase,
	CCTTATAGCTGGGAACGCTAGG	antisense	alternatively spliced
Ago61	CAAACACACCTGTCACTCAGTT	sense	ESTs, Highly similar to hypothetical
	CTTAGCAGTGAGGAGGAGGC	antisense	protein FLJ14566 [Homo sapiens]
Pdcd8	TTGTTCGATTCCTGCTCCTACA	sense	programmed cell death 8
	AAGACGGTGAGCAACATGAAGA	antisense	(apoptosis-inducing factor)
kdr	CCTTCGAGTTTCTCAACGTCCT	sense	FLK1 kinase insert domain
	TTTGGTACCATAGCGTGACGAG	antisense	receptor (VEGF receptor 2)

Real Time-PCR Analysis of Identified Gene Related with Hepatotoxicity

We performed quantitative real time PCR analysis to confirm the differential expression of identified gene which have specific expression pattern related with each chemical treatment. Three up regulated genes related with TAM treatment, Pcdk 1, Cst K and Cst E and three down regulated genes related with MTX treatment, Pdcd 8, ago61 and kdr were examined. All these genes were same result compare with microarray data.

Discussion

In this report we would like to know whether generation of chemical treatment related gene expression profiles, using microarray analysis, would permit classification of chemical associated phenotype. Tamoxifen (TAM), a non-steroidal antiestrogen anticancer drug and chemopreventive agent for breast cancer, have caused cholestasis in liver. The potent hepatocarcinogenicity of this drug has been reported. Methotrexate (MTX) is dihydrofolate reductase inhibitor which interfaces with the synthesis for urine nucleotide and dTMP. And it may cause atrophy, necrosis and steatosis in liver. These two anticancer drug have well-known hepatotoxicity. So, we generated gene expression profiles for two anticancer drugs. Each anti-cancer drug have different action mechanism and specific hepatotoxicity. And they have specific gene expression pattern related with their own toxicological phenotype compared with other toxicological and toxicogenomics study. Gene expression profiling of MTX high dose treatment group showed that 10 genes were up regulated, e.g, Hadha (long fatty acid oxidation related gene), DNAi (inhibitor of DNA binding 3), Cal III (calmodulin III). And in these group 14 genes were down regulated, e.g, Cts E (cathepsin E), Cts K (cathepsin K), TFIIA, Pctk 1 (Cdk relate kinase family), Lyp III (Lipophospholipase II). And MTX low dose group showed that 9 genes were down regulated, e.g, EST-cal (similar to S50193 Ca^{2+} /calmodulin-dependent protein kinase). In the case of TAM high dose treatment group, 10 genes were up regulated, e.g, Kdr (VEGF 2 receptor), Pdcd 8 (apoptosis-inducing factor), PRKC (apoptosis related gene) and 4 genes were down regulated, e.g, EST-SY21 (similar to SY21_MOUSE Small inducible cytokine A21). And TAM low dose group showed that 8 genes were up regulated, e.g, kdr, BMP-6 and 3 genes were down regulated., e.g, EST-cc (similar to serologically defined colon cancer antigen 28). TAM treatment induced apoptosis in breast cancer[16]. In this study, TAM treatment group showed up-reglulated expression profile with apoptosis related gene, Pdcd 8 and Prkc. And the gene expression profile related with antitumor activity also were showed, e.g, kdr (VEGF 2 receptor)[17] in TAM treatment and Cst K (cathepsin K)[18] in MTX treatment. So, these may be useful data for toxicogenomic approaching of these kind of drug. And we will try to deeply approach to find the function of EST related with these trial.

As these results, we have shown that DNA microarray technology could be useful tool for studying changes in gene expression profiles due to drug treatments. And there are reasonable relationship between gene expression profiles and toxicological effect. But the link between the gene expression profile and toxic or undesired effect of drug on animals or human is not completely finished and will require the use of cluster analysis of various kind of drugs. But no doubt that the technology of DNA microarray, toxicogenomics, will serve as a screening tool in drug discovery or early pharmaceutical development.

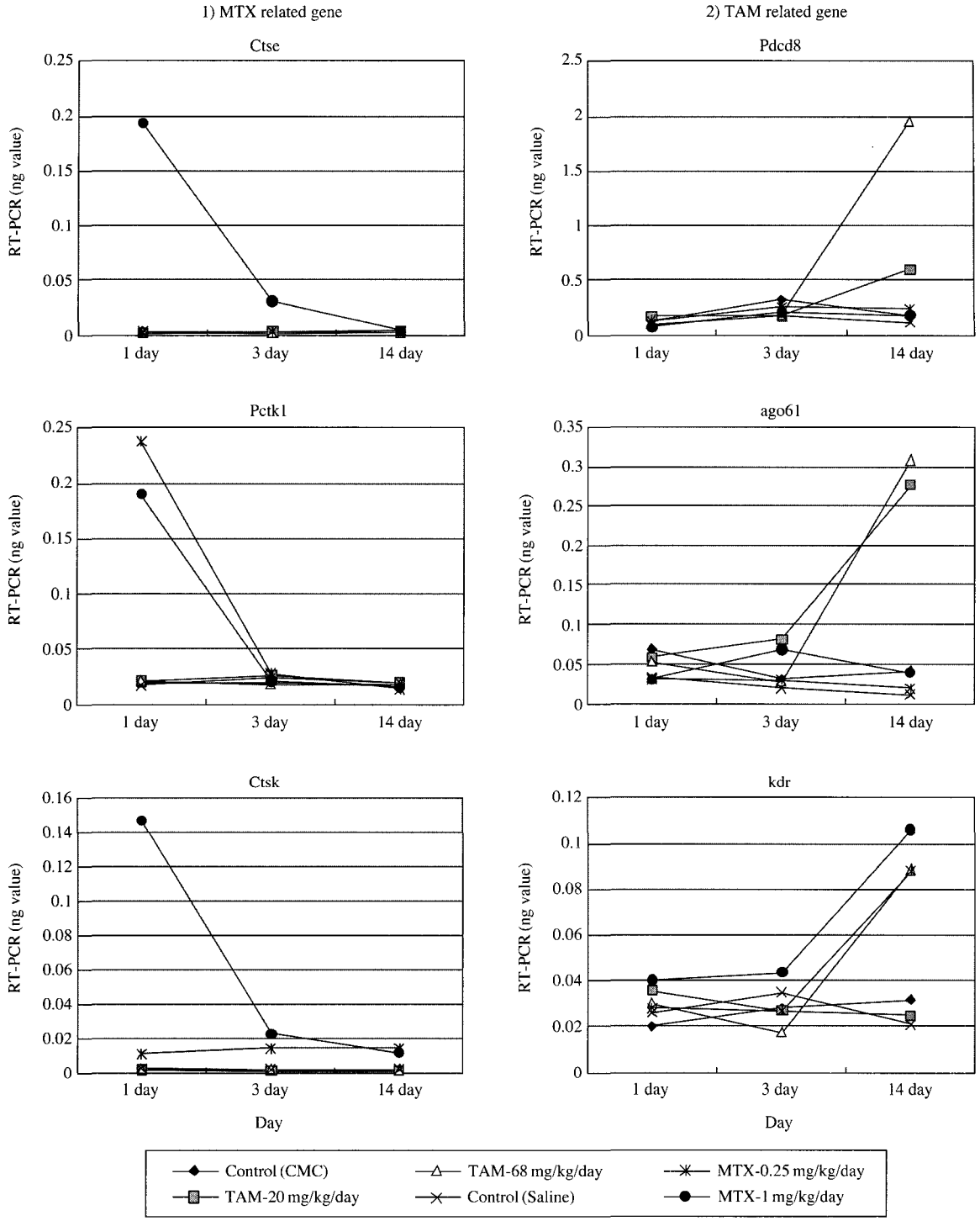

Fig. 1. Real Time-PCR analysis of identified gene related with hepatotoxicity.

Methods

Materials

Tamoxiphen (TAM, CAS #, 99% pure) and Methotrexate (MTX, CAS #59-05-2, 99% pure) were obtained from IFFECT CHEMPHAR Co., Ltd. (China). Corn oil were obtained from Sigma-Aldrich Co. (USA). The 4.8 K cDNA chips used in this study

were constructed at the GenoCheck, Co., Ltd. Which were already developed for toxicogenomics study (Ansan, Korea)[5].

Animal Treatment, Sample Collection, and Histopathological Analysis

Male Sprague-Dawley CD rats (Crl: CD (SD) IGS BR, aged at 5 weeks) obtained from Orient Bio Co. Ltd. were weighted, weight-ranked and assigned randomly to each of the treatment and control group before administration and then housed group (4 rats/group) in polycarbonate, wooden chip provided cages (Jang-Do BNC, Korea) during the study. Rats were allowed certified Rodent diet (Picolab® Rodent diet 5053) and water *ad libitum*. The animals were kept under controlled lighting (12 hr light-dark cycle), temperature (22°C ± 2.5°C), a relative humidity (55 ± 10%) and were acclimated to this environment for 7 days prior to the start of the study. All animals were randomly assigned to treatment group. TAM was diluted with Corn oil (low dose at 20 mg/kg and high dose at 68 mg/kg as TAM concentration) for 1, 3, 7 and 14 days. TAM treatment group was received the drug by orally administration. And Control group was received corresponding quantities of sterile corn oil by same route. And MTX was diluted with sterile saline (low dose at 0.25 mg/kg and high dose at 1.0 mg/kg as MTX concentration) for 1, 3, 7, 14 days. MTX treatment group was received the drug by orally administration. And also control group was received the same quantities of sterile saline by same route. The doses were selected based on review of literature that produced hepatotoxicity without lethality after the desired exposure period. During the all period, body weight and food consumption of all animals were measured weekly. All animals were checked 2 times daily (generally after oral administration) for signs of toxicity. Based on the most recently recorded body weights, the volume of drug administered was adjusted. Following treatment, all animals of each group were sacrificed by CO_2 gas and the livers were removed immediately for histopathology and total RNA purification. And at this time, we also have collected the blood sample for blood chemistry analysis and hematology. Left lateral lobe of the liver was sliced (about 5 mm) in cross-secretion close to the center of the lobe and immediately placed in RNA Later (Ambion, TX) for RNA extraction and gene expression analysis. The samples were stored at −70 °C until further processing. The remaining portions of liver were collected in 10% neutral buffered formalin for histopathologic evaluation. At necropsy, liver weights were recorded. The percent ratios of organ to body weight were determined.

The liver tissues collected in formalin at necropsy were processed embedded in paraffin, sectioned at 5 micros, and stained with hematoxylin and eosin (H & E). Histopathologic examinations of liver tissues were conducted by skillful pathologist.

Statistical Analysis for *in vivo* Study

Significant differences between means were identified using a multifactor ANOVA. The student's *t*-test was used for one-to-one comparisons versus a control group. Probability of significance was set at $P < 0.05$. All data are reported as mean ± S.D.

Generation of cDNA Array

The 4.8 K cDNA chip made in house at Genocheck. Co., Ltd (Ansan, Korea) was used for gene expression profiling induced by chemicals especially for hepatotoxicity[5]. The list of the genes on used microarray is able to gain from Web site (http://www.genocheck.co.kr) (Table 1). The selected genes were involved in basic cellular process such as drug metabolism, stress responses, cell proliferation, cell cycle activation, transcription, inflammation and apoptosis. To evaluate the reliability of experimental data, several controls of positive and negative hybridization and detection control were included on this chip.

RNA Preparation and Probe Labeling

Total RNA was then extracted from liver tissues using a RNeasy mini Total RNA Purification kit (Qiagen. Inc.). Liver sections of 50 mg were used for total RNA isolation. The Lysis buffer was added to frozen liver sections and the tissue was immediately homogenized using a DIAX 900 Homogenizer (Heidolph). All processes were followed by manuals of manufacture. Final product yielded 260 nm/280 nm ratios of 1.8-2.0, purity was confirmed via gels and concentration was determined based on 260 nm absorbencies using a spectrophotometer. The quality of the RNA was checked by visualization of the 28S : 18S ribosomal RNA ratio on a 1% agarose gel.

For microarray hybridizations, each total RNA sample (50 μg) was labeled with Cyanine 3 (Cy3)-or Cyanine 5 (Cy5)-conjugated dCTP (Amersham) by reverse transcription using reverse transcriptase, SuperScript™ II (Invitrogen), positive control RNAs (0.5 ng) and 2 μg oligo dT primer (Genotech., Korea). Whole processes were following as manufacture's manuals (Invitrogen).

cDNA Microarray Hybridization, Scanning of Signals and Microarray Data Analysis

The fluorescent labeled cDNA were mixed and hybridized simultaneously to the 4.8 K cDNA chip

for overnight at 42°C. The cDNA chips were washed, dried and scanned with an Axon Scanner Genefix 4000B (Axon Instruments) using independent laser excitation of 2 fluors at 532 nm and 635 nm wavelengths for Cy3 and Cy5 labels, respectively. The raw pixel intensity images were analyzed using the GenePix Pro 4.0 software (Axon Instruments). The fluorescent intensity of each DNA spot (average of intensity of each pixel present within the spot) was calculated using local mean background subtraction.

The data log 2 transformed and transferred the information to Microsoft Access. Overall differences in gene expression between samples were calculated by a within-slide normalization for location and scale methods for all of the expressed genes on the array[12]. For each of the arrays for each group, genes were included for additional analysis if the log 2 ratio was ± 0.8 or more for at least two of the three arrays. To investigate differential gene expression profiles between two agents, genes were rank-ordered and selected according to a Modified Wilcoxon test and between-groups to within-groups sum of squares (BSS/WSS) criterion [13-15].

Real Time-PCR Test

For confirm the gene expression profile detected by cDNA microarray data, we use the real time PCR.

For quantitative RT-PCR, total RNA was isolated from the liver tissue which was obtained from each treated animal using a RNeasy mini Total RNA Purification kit (Qiagen. Inc.) followed the recommendations of manufacturer. Total RNA (2 μg) was used to generate cDNA in each sample using the SuperScript II RT with oligo (dT) 16 primer. Aliquots (5-10%) of total cDNA were amplified in each PCR reaction mixture that contains 0.5 μM of sense and antisense primers of selected genes (Table 3). The RT-PCR reactions were carried out in a MJ Research themocycler DNA Engine. The purified total RNA were performed reverse-transcription into cDNA by using an QuantiTect SYBR Green RT PCR kit (Qiagen). The real time RT-PCR was performed by using a QuantiTect SYBR Green master mix (Qiagen), and running for 45 cycle at 95°C for 20 sec and 60°C for 1 min. The PCR efficiency was examined by serially dilution of the λ DNA and the melting curve data were collected to check the PCR specificity. Each sample was triplicated and the corresponding no-cDNA sample was included as a negative control.

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