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Identification of Gene-based Potential Biomarkers for Cephalexin-induced Nephrotoxicity in Mice

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Abstract

Cephalexin, one of most widely prescribed cephalosporin, has been reported to cause acute renal failure as a side effect in human and experimental animals. Although numerous animal studies have been reported for the cephalosporin nephrotoxicity, the molecular and cellular nephrotoxic mechanisms of cephalexin are still unknown. This investigation evaluated the time-dependent gene expression profile of kidney in mouse during cephalexin induced nephrotoxicity. C57BL/6 female mice were administered either saline or 1,000 mg/kg cephalexin intraperitoneally. Mice were sacrificed at 3, 6, and 24 hr after administration. Blood biochemical and histopathological results indicated cephalexin induced nephrotoxicity. Microarray experiment carried out using Affymetrix GeneChip®. There were 198 informative genes that were significantly expressed >5-fold versus control at 3, 6, and 24 hr ($p<0.01$), of which 156 and 42 were up- and down-regulated, respectively. Major classes of up-regulated genes at 3, 6 hr included those involved in MAPK/Jak-STAT signaling pathway and immune response such as cytokine-cytokine receptor interaction and complement and coagulation cascades. At 24 hr, up-regulated genes were mainly involved in regeneration /repair and immune response; down-regulated genes were generally associated with transporters and intermediary metabolism. Among the up-regulated genes at 24 hr, several potential biomarkers on nephrotoxicity such as *Kim-1, Fga, Timp1*, and *Slc34a2* were clustered in a same category. In addition, *Tnfrsf12a* and *Lcn2* which were consistently up-regulated (>5 fold) were also included as potential biomarkers. These results may provide clues for elucidating the mechanism of cephalexin induced nephrotoxicity and evaluating potential biomarkers to assess nephrotoxicity.

Keywords: Cephalexin, Nephrotoxicity, Biomarker, Gene expression analysis

Cephalexin, is the first generation of cephalosporin antibiotics which has β-lactam ring structure, has been widely used as oral cephalosporin especially in the treatment of respiratory and urinary tract infections[1,2]. Cephalosporin antibiotics have been reported to induce acute renal failure as a side effect when given in a large single dosage in human as well as experimental animals. The nephrotoxic cephalosporin antibiotics cause acute proximal tubular necrosis prominently on S2 segment[3]. The mechanism of renal injury mainly developed by secretary uptake of the antibiotics by tubular cell, acylation of target protein, and reactive oxygen species (ROS)-induced lipid peroxidation[4]. The nephrotoxic effects of most cephalosporin antibiotics are correlated with their accumulation in renal cortex[4]. However, cephaloglycin and cephalexin have rapid excretion property in the tubular cell compare to other cephalosporin antibiotics, but have very different nephrotoxic potential[5]. As a result of more reactive side chain, acetoxyl living group, cephaloglycin has known for 9 times more reactive than cephalexin. Although cephalosporin antibiotics have common β-lactam ring, their nephrotoxic initiation mechanism and their pharmacokinetic properties are depend on their side-group substituents[5]. Although there has been extensive progress to elucidate nephrotoxic mechanism of cephalosporin induced renal injury, most studies are focused on specific cephalosporin antibiotics and the mechanism of cephalexin induced nephrotoxicity is still obscure. Furthermore, only a few toxicogenomic approaches using microarray technology to assess the nephrotoxicity of cephalosporin antibiotics have been reported.

Gene expression analysis using DNA microarray plays an important role as a sensitive indicator of toxicant exposure, disease state, and cellular metabolism. The characterization of gene expression profiling upon exposure to toxicants can both provide information about the mode of action of toxicants and a kind of "genetic signature" from the pattern of gene

expression changes[6,7]. The development of such gene expression signatures could enable DNA microarray to allow rapid screening of unknown toxicants since the application of DNA chips to toxicology was proposed in the late 1990s[8]. These applications have evaluated the potential of the technology[9], and gene

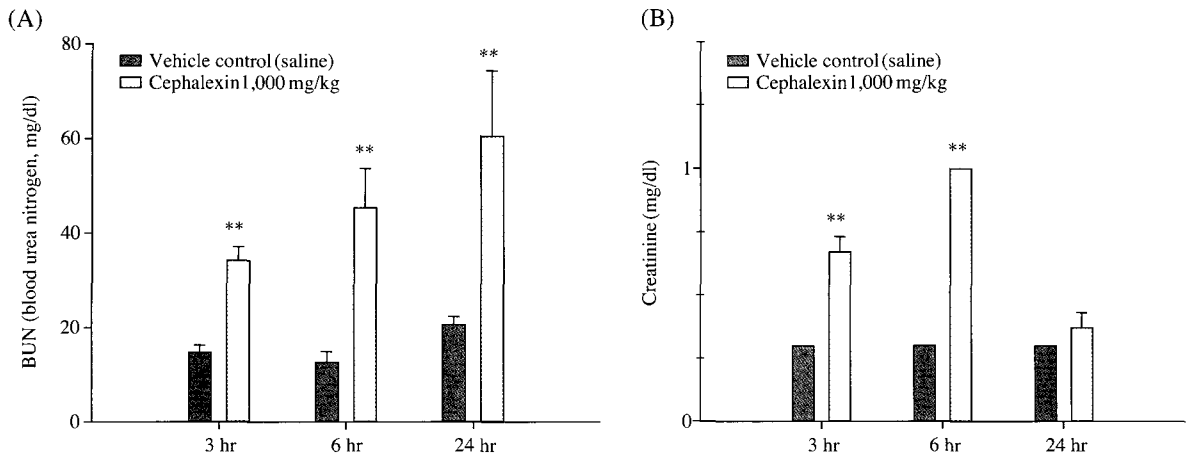

Fig. 1. Serum BUN (A) and creatinine (B) level at 3, 6, and 24 hrs following treatment with cephalexin. Each bar represent Mean ± SD (n=3) obtained from 3 animals in each time group, $^{**}p<0.01$, compare with the control.

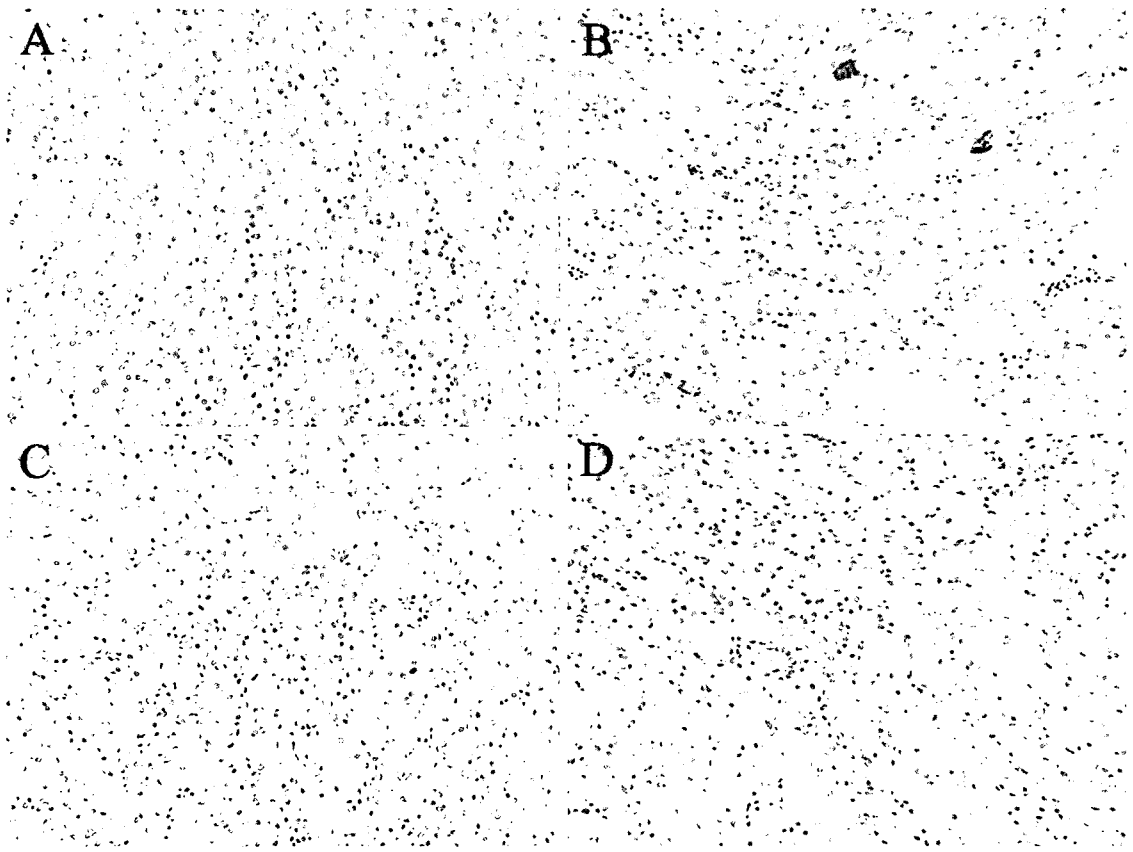

Fig. 2. Light photomicrographs of kidneys from treatment with vehicle control (A) and 1,000 mg/kg cephalexin (B-D). All tissues were stained with H&E (×200). (A) Control group, (B) 3 hr group, (C) 6 hr group, (D) 24 hr group.

expression profiling practically has been used in toxicological assessments[7,10]. The field of toxicogenomics has rapidly progressed to actual applications, and gene expression profiling is now being used in evaluation for toxicity of chemical compounds[11].

In this study, we applied toxicogenomic tools to elucidate cephalexin induced gene expression and infer potential candidates for nephrotoxicity marker based on biochemical, histopathological, and pharmacokinetic information. Cephalexin has known as comparably mild nephrotoxic among cephalosporin antibiotics[12], but it has advantage for assessment acute nephrotoxicity since it has rapid absorption, excretion property, and short half-life time. The aim of this study was to conduct potential nephrotoxic markers after verification of the relationship between conventional toxicology result and gene expression profiling.

Biochemical and Histopathological Examination

We measured serum blood urea nitrogen (BUN) and creatinine to determine cephalexin induced nephrotoxicity. Mice administered with cephalexin (1,000 mg/kg) showed significantly increased serum BUN level at 3, 6, and 24 hr groups compare to their control groups. In case of serum creatinine, higher increase was shown at 3, 6 hr groups, but slightly increased at 24 hr group (Fig. 1).

In light microscopic observation, medullar and tubular cast and tubular dilation were examined at all time points but tubular vacuolation was observed only at 6 hr group. The degeneration such as tubular cast and tubular dilation was increase at 24 hr group compare to 3, 6 hr group. In view of the results, we estimated that cephalexin had influence on kidney moderately (Fig. 2).

Gene Expression Analysis

We used Affymetrix GeneChip® to analyze the gene expression profiles of mouse kidneys which were affected by cephalexin. Gene expression changes were analyzed by comparing treated group versus control group at each time point using a statistical criteria of $\geq$5-fold changes at $p<0.01$, respectively. In this analysis, we identified 198 genes that were significantly changed more than 5-fold in at least one of the time points. One hundred fifty-six genes were up-regulated and 42 genes were down-regulated.

To identify mechanistic changes according to the time points, significantly changed 198 genes were analyzed using two-dimensional (2-D) hierarchical clustering[13] (Fig. 3). The results of hierarchical clustering showed that the expression profiles of cepha-

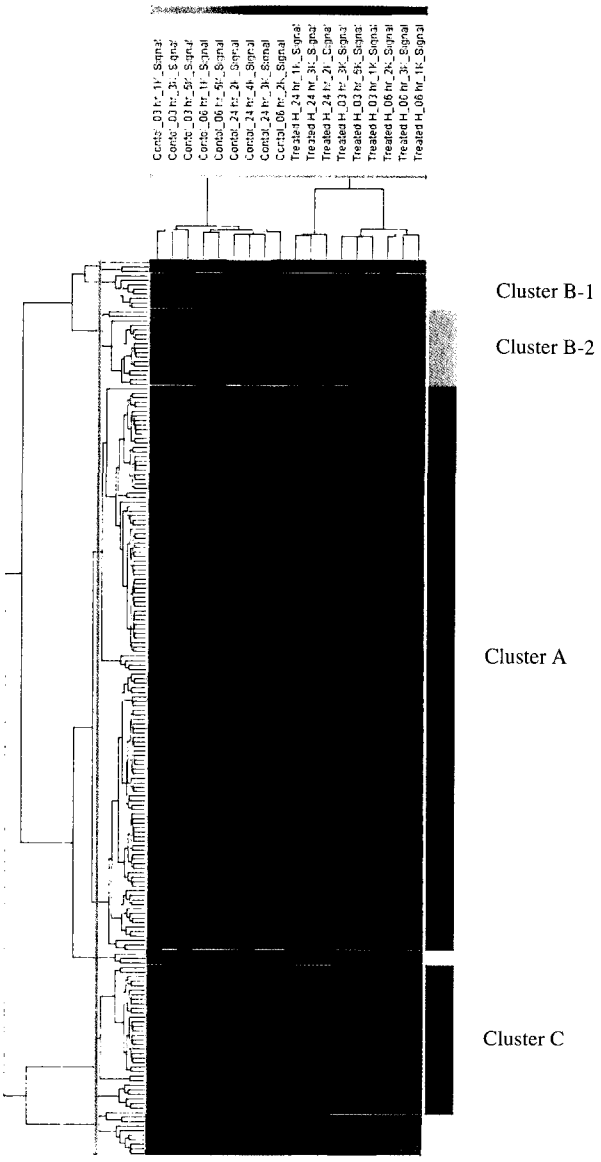

Fig. 3. Hierarchical clustering of five fold differently expressed genes (198 genes) in kidneys after cephalexin treatment ($p<0.01$). The genes of each time points were exhibited different expression profiles. Each color branch represents the classified categories: cluster B-1 (yellow), cluster B-2 (green), cluster A (red), cluster C (blue).

lexin treated animals were generally grouped in a time-dependant manner. There were no significant differences between 3 and 6 hr group, but 24 hr group has quite different gene expression profiles compare to 3 and 6 hr groups. Principal components analysis also suggested discrepancies between control and 3, 6, 24 hr group (Fig. 4).

In this experiment, hierarchical clustering analysis formed three major groups: control, 3, 6, and 24 hr

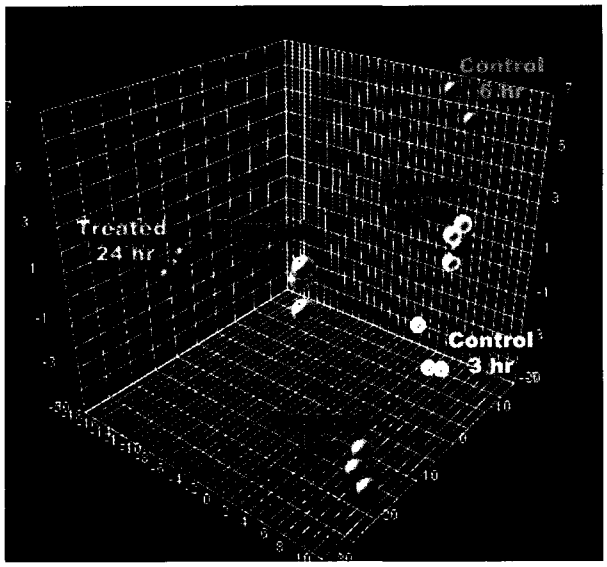

Fig. 4. Principal Components Analysis (PCA) using five fold differentially expressed genes (198 genes) ($p<0.01$).

group. Since cephalexin has comparably short half-life time (0.5-1.2 hours) and rapid absorption and excretion properties, it was noted that gene expression changes were closely associated with its pharmacokinetic properties. Therefore, grouping of animals based on gene expression changes depend on time-manner suggests that gene expression changes were indexes of elucidating mechanism of toxicity. Based on this paradigm, we focused on the genes which had considerably different gene expression profiles between early and late time groups. We classified genes to three main clusters, cluster A, cluster B, and cluster C, according to their expression patterns (Fig. 3). The gene expression level of cluster A was increased at 3, 6 hr groups and keep their expression level or return to the normal range at 24 hr group. These genes were classified according to KEGG pathway to analyze molecular mechanism during nephrotoxicity. Most genes of cluster A involved in cytokine-cytokine receptor interaction, MAPK signaling pathway and Jak-STAT signaling pathway (Table 1). On the

Table 1. The genes of cluster A were classified by KEGG pathway.

Gene title	Gene symbol	Acc. No.	Fold change		
			3 hr	6 hr	24 hr
MAPK signaling pathway					
dual specificity phosphatase 8	*Dusp8*	NM_008748	3.3	2.3	1.3
nuclear receptor subfamily 4, group A, member 1	*Nr4a1*	NM_010444	1.8	2.5	−0.8
DNA-damage inducible transcript 3	*Ddit3*	NM_007837	2.8	3.1	0.3
growth arrest and DNA-damage-inducible 45 alpha	*Gadd45a*	NM_007836	3.0	3.1	1.2
growth arrest and DNA-damage-inducible 45 beta	*Gadd45b*	NM_008655	2.4	2.3	0.1
activating transcription factor 4	*Atf4*	NM_009716	2.3	2.3	1.0
FBJ osteosarcoma oncogene	*Fos*	NM_010234	4.7	5.0	1.0
heat shock protein 1	*Hspb1*	NM_013560	2.0	2.4	1.7
Cytokine-cytokine receptor interaction					
chemokine (C-X-C motif) ligand 1	*Cxcl1*	NM_008176	5.3	6.3	3.0
tumor necrosis factor receptor superfamily, member 12a	*Tnfrsf12a*	NM_013749	4.2	4.7	3.5
chemokine (C-C motif) ligand 2	*Ccl2*	NM_011333	4.5	3.2	2.6
colony stimulating factor 1 (macrophage)	*Csf1*	NM_007778	1.8	2.4	1.3
chemokine (C-X-C motif) ligand 14	*Cxcl14*	NM_019568	1.8	2.5	0.9
inhibin beta-B	*Inhbb*	NM_008381	3.3	3.3	1.3
interleukin 6	*Il6*	NM_031168	6.2	6.8	4.8
Jak-STAT signaling pathway					
leukemia inhibitory factor	*Lif*	NM_008501	4.6	5.4	3.5
oncostatin M receptor	*Osmr*	NM_011019	2.3	2.6	2.0
interleukin 11	*Il11*	NM_008350	0.9	3.0	2.2
suppressor of cytokine signaling 3	*Socs3*	NM_007707	4.6	4.9	3.7
Wnt signaling pathway					
fos-like antigen 1	*Fosl1*	NM_010235	6.5	7.5	4.1
Jun oncogene	*Jun*	NM_010591	2.3	2.5	1.0
myelocytomatosis oncogene	*Myc*	NM_010849	3.8	4.2	1.8
Complement and coagulation cascades					
coagulation factor III	*F3*	NM_010171	3.3	2.7	1.7
plasminogen activator, urokinase receptor	*Plaur*	NM_011113	3.0	2.9	1.4
serine (or cysteine) peptidase inhibitor, clade E, member 1	*Serpine1*	NM_008871	4.4	4.0	3.5

The relative fold change of treated group versus control was calculated at each time point and presented as log base 2. ($p<0.01$, Welch's t-test)

Table 2. Up-regulated genes at 24 hr after cephalexin treatment.

Gene title	Gene symbol	Acc. No.	Fold change		
			3 hr	6 hr	24 hr
CLUSTER B-1					
Proliferation					
CDC28 protein kinase 1b	*Cks1b*	NM_016904	0.2	0.6	2.6
minichromosome maintenance deficient 5, cell division cycle	*Mcm5*	NM_008566	−0.5	−0.7	2.6
Immune/Inflammation					
complement component 3	*C3*	NM_009778	−0.3	0.2	2.4
T-cell immunoglobulin and mucin domain containing 2	*Timd2*	NM_134249	−0.9	0.4	3.4
Intermediary metabolism					
UDP glucuronosyltransferase 2 family, polypeptide B5	*Ugt2b5*	NM_009467	−1.1	−2.3	3.6
Regeneration/repair					
procollagen, type III, alpha 1	*Col3a1*	NM_009930	0.3	0.8	2.5
ubiquitin-like, containing PHD and RING finger domains, 1	*Uhrf1*	NM_010931	−0.8	−0.5	4.4
CLUSTER B-2					
Immune/inflammation					
interleukin 1 family, member 6	*Il1f6*	NM_019450	0.9	2.4	3.5
interleukin 1 receptor antagonist	*Il1rn*	NM_031167	2.9	2.7	1.4
CD68 antigen	*Cd68*	NM_009853	0.7	1.8	2.4
Intermediary metabolism					
aldehyde dehydrogenase family 1, subfamily A2	*Aldh1a2*	NM_009022	0.6	1.9	2.8
peptidyl-tRNA hydrolase 1 homolog (S. cerevisiae)	*Ptrh1*	NM_178595	0.6	1.6	2.8
sphingomyelin phosphodiesterase, acid-like 3B	*Smpdl3b*	NM_133888	0.7	0.3	3.0
Cytoskeleton/cell morphology					
annexin A3	*Anxa3*	NM_013470	1.0	1.9	2.4
S100 calcium binding protein A6 (calcyclin)	*S100a6*	NM_011313	1.3	1.9	2.5
Proliferation					
gastrin releasing peptide	*Grp*	NM_175012	−0.2	3.4	4.4
Regeneration/repair					
fibrinogen, alpha polypeptide	*Fga*	NM_010196	2.3	3.5	3.8
fibrinogen, gamma polypeptide	*Fgg*	NM_133862	1.5	3.4	4.9
hepatitis A virus cellular receptor 1	*Havcr1*	NM_134248	0.9	4.2	8.6
serine (or cysteine) peptidase inhibitor, clade A (alpha-1 antiproteinase, antitrypsin), member 10	*Serpina10*	NM_144834	0.2	1.5	3.7
tissue inhibitor of metalloproteinase 1	*Timp1*	NM_011593	3.5	4.2	4.9
Transporter					
solute carrier family 34 (sodium phosphate), member 2	*Slc34a2*	NM_011402	1.0	1.1	2.3
Miscellaneous					
DNA segment, Chr 17, human D6S56E 5	*D17H6S56E-5*	NM_033075	1.1	1.4	2.5
DNA segment, Chr 17, human D6S56E 5	*D17H6S56E-5*	NM_033075	1.1	2.2	2.7

Inclusion in the table was based on ≥5-fold up-regulation at 24 hr compare to control. The relative fold change of treated group versus control calculated at each time point and presented as log base 2. ($p<0.01$, Welch's t-test)

other hand, the genes of cluster B and C were highly up- and down-regulated at 24 hr group, respectively (Fig. 3). The genes of cluster B and C were listed Table 2 and 3. In contrast to the genes in cluster A, B, and C, several genes such as TNF receptor superfamily, member 12a (*Tnfrsf12a*), methylenetetrahydrofolate dehydrogenase (*Mthfd2*), lectin, galactose binding, soluble 3 (*Lgals3*), lipocalcin 2 (*Lcn2*), TSC22 domain family, member 1 (*Tsc22d1*) and suppressor of cytokine signaling 3 (*Socs3*) were up-regulated and solute carrier family 4 (anion exchanger), member1 (*Slc4a1*) was down-regulated more than five folds at all time points, respectively.

Discussion

The results of this study demonstrate that cephalexin treatment results in histopathological and blood biochemical evidence of nephrotoxicity and also induces a remarkable alteration of the gene expression profiles by time-dependant manner in C57BL/6 mice. In the analysis of histopathological data in kidney, medullar and tubular cast and tubular dilation were observed at all time points but tubular vacuolation was observed only at 6 hr group. In general, cephalosporin antibiotics induce proximal tubular necrosis[14].

Table 3. Down-regulated genes at 24 hr after cephalexin treatment.

Gene title	Gene symbol	Acc. No.	Fold change		
			3 hr	6 hr	24 hr
Calcium homeostasis					
calbindin-28K	*Calb1*	NM_009788	−1.0	−1.5	−2.5
parvalbumin	*Pvalb*	NM_013645	−0.8	−1.0	−3.9
Immune response					
lymphocyte antigen 6 complex, locus F	Ly6f	NM_008530	0.1	−0.3	−2.4
secreted and transmembrane 1	Sectm1	NM_026907	−0.7	−1.5	−2.8
WAP four-disulfide core domain 15	*Wfdc15*	NM_138685	−0.5	−0.8	−2.4
Intermediary metabolism					
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	*Hmgcs2*	NM_008256	0.0	−1.7	−4.5
RIKEN cDNA 1810022C23 gene	*1810022C23Rik*	NM_026947	−0.3	−0.6	−2.5
aminoacylase 1	*Acy1*	NM_025371	−0.1	−1.0	−2.4
aldolase 3, C isoform	*Aldoc*	NM_009657	−0.6	−1.1	−2.4
expressed sequence AU018778	*AU018778*	NM_144930	0.0	−0.2	−2.7
3-hydroxybutyrate dehydrogenase, type 1	*Bdh1*	NM_175177	−0.2	−1.1	−3.2
3-hydroxybutyrate dehydrogenase, type 1	*Bdh1*	NM_175177	−0.5	−0.7	−2.6
betaine-homocysteine methyltransferase	*Bhmt*	NM_016668	−0.4	−1.0	−3.1
carboxylesterase 3	*Ces3*	NM_053200	0.0	−0.4	−2.6
indolethylamine N-methyltransferase	*Inmt*	NM_009349	−0.4	−0.8	−2.9
meprin 1 beta	*Mep1b*	NM_008586	−0.4	−0.7	−3.3
Oxidative stress					
RIKEN cDNA 9030605E09 gene	*9030605E09Rik*	NM_201360	−0.7	−0.8	−2.5
aldo-keto reductase family 1, member C14	*Akr1c14*	NM_134072	0.0	−0.8	−5.0
aldo-keto reductase family 1, member C18	*Akr1c18*	NM_134066	−0.5	−0.1	−2.4
glutathione peroxidase 6	*Gpx6*	NM_145451	−0.2	−0.5	−2.5
4-hydroxyphenylpyruvic acid dioxygenase	*Hpd*	NM_008277	−0.3	−1.1	−4.1
Proliferation					
epidermal growth factor	*Egf*	NM_010113	−0.3	−0.5	−2.9
inhibitor of DNA binding 4	*Id4*	NM_031166	−1.4	−1.9	−2.9
Transporter					
solute carrier family 17 (sodium phosphate), member 2	*Slc17a2*	NM_144836	−0.6	−0.5	−2.6
solute carrier family 22 (organic anion transporter), member 7	*Slc22a7*	NM_144856	−0.1	−0.4	−3.0
solute carrier family 25 (mitochondrial carrier, phosphate carrier), member 25	*Slc25a25*	NM_146118	0.5	0.6	−2.7
solute carrier family 2 (facilitated glucose transporter), member 5	*Slc2a5*	NM_019741	−0.2	−0.9	−2.4
Xenobiotics metabolism					
cytochrome P450, family 2, subfamily d, polypeptide 9	*Cyp2d9*	NM_010006	−0.1	−1.2	−2.6
Miscellaneous					
expressed sequence AV344025	*AV344025*		−1.5	−2.0	−2.5
nucleosome assembly protein 1-like 5	*Napl15*	NM_021432	−0.5	−0.5	−3.5
polymerase (RNA) III (DNA directed) polypeptide E	*Polr3e*	NM_025298	−0.3	−0.8	−2.5

Inclusion in the table was based on ≥5-fold down-regulation at 24 hr compare to control. The relative fold change of treated group versus control calculated at each time point and presented as log base 2. ($p<0.01$, Welch's t-test)

However, no signs of apoptosis and necrosis were observed in this experiment. Serum BUN and creatinine levels did not correlate with histopathological alteration. BUN level was increased time dependently, but creatinine level was decreased at 24 hr group compare to 3, 6 hr group. The rapid elimination rate of cephalexin through urinary excretion suggests that decreased creatinine level at 24 hr. Although serum BUN and creatinine have been commonly used as markers to assess nephrotoxicity, there is a need of early detectable biomarkers to assess nephrotoxicity. Because they are relatively insensitive and their levels are only be elevated when more than 75% of kidney cells are no longer functioning[15].

We used Affymetrix GeneChip® to investigate gene expression changes in the kidney following cephalexin administration and to identify potential biomarkers to assess nephrotoxicity. We divided genes into three major clusters to analyze their expression patterns according to 2-D hierarchical clustering results. The genes included cluster A were highly up-regulated at 3, 6 hr and were kept up their expression level

or returned to normal range at 24 hr. The majority of those genes were involved in MARK signaling pathway, Jak-STAT signaling pathway, cytokine-cytokine receptor interaction and complement and coagulation cascades. MAPK signaling pathway is modulated by reactive oxygen species (ROS)[16], and is associated with hormone and chemical induced nephrotoxiciy[17,18]. Jak-STAT signaling pathway is major signaling pathway converting the cytokine signal into gene expression programs regulating the proliferation and differentiation of the immune cells[19]. These results suggest that cephalexin induced nephrotoxicity is associated with the response against ROS and the up-regulated genes at 3 and 6 hr group were strongly related with initial event of acute toxicity. Up-regulation of genes involved in these signaling pathways also suggests that the genes involved in immune response should be triggered by cluster A gene set, then be participated in immune and inflammation responses. Consequently, the up-regulated genes at 24 hr group compare to 3 and 6 hr groups (cluster B) were mainly involved in inflammation response, regeneration/repair, cell cycle (proliferation) and intermediary metabolism. Recent studies based on gene expression analysis showed that genes involved in inflammation, regeneration and repair were up-regulated at 24 hr and they tend to last their expression level[20,21]. Furthermore, tissue repair processes are a principle determinant of the progression of renal injury whether acute renal injury is restored or proceeds to irreversible severe necrosis, renal failure, and death[20]. Therefore, it is suggested that the genes significantly up- and down-regulated at that time point be considered as molecular markers to represent nephrotoxicity.

To date, several modulated genes in response to renal injury induced by a diverse group of model nephrotoxicants including, cisplatin[22], gentamycin[22,23], puromycin[22,24], amphotericin[24], mercuric chloride, and fluoromethyl-2, 2-difluoro-1-(trifluoromethyl)-vinyl ether[21] were identified. Those genes were highly up-regulated ($\geq$5-fold) and significantly rather consistently, include glutathione transferase pi, kidney injury molecule-1 (*Kim-1*), *osteopontin*, tissue inhibitor of metalloproteinase 1 (*Timp1*), *clusterin, vimentin*, and fibrinogen alpha (*fga*). Significant down-regulated genes ($\geq$5-fold) include generally *regucalin, Slc21/22* transporter families and heat shock protein 67 (*Hsp67*). In addition, insulin-like growth factor binding protein-1 (*Igfbp-1*), fibrinogen alpha (*fga*), Glutathione transferase alpha (*Gst-a*), lipocalcin (*Lcn*), TNF receptor superfamily, member 12a (*Tnfrsf12a*), and several transporters (*Slc21a2, Slc15, Slc34a2*) were identified as potential biomarkers[22-24].

Among the genes in cluster B-2, *Kim-1*, has been known as an early biomarker on nephrotoxicity in human and rodent model, was up-regulated more than 300 fold. The precise role of *Kim-1* in nephrotoxicity is unknown, but it is up-regulated in an association with differentiated and regeneration tubular epithelial cell in response to several renal ischemia and toxicants and is participated in repair processes involving dedifferentiation, migration, proliferation, and restoration of cellular structure and function[25,26]. Interestingly, as well as *Kim-1*, several candidate biomarkers on nephrotoxicity such as *Fga, Timp1*, solute carrier family 34 (*Slc34a2*) were clustered in a same category (cluster B-2). In down-regulated genes at 24 hr group (cluster C), several transporters (*Slc17a2, Slc22a7, Slc25a25, Slc2a5*) were observed. In previous reports, transporters and transport mechanism related genes were often damaged during acute renal injury because they contribute to the absorption and/or excretion of drugs, xenobiotics, and endogenous compounds in the intestine, liver, and kidney[27]. In contrast to several transporters were down-regulated during renal injury, *Slc34a2* was only up-regulated. This finding is similar to that of gene expression patterns results from several other nephrotoxicants[21,24]. Finally, *Tnfrsf12a* and *Lcn2* which were consistently up-regulated more than five folds in this experiment were also included as potential biomarkers.

In conclusion, our results showed that the changes of gene expression patterns were associated with nephrotoxicity induced by cephalexin. The gene expression patterns of several up-regulated genes included *Kim-1, Fga, Timp1, Slc34a2, Tnfrsf12a*, and *Lcn2* coincided with those of potential biomarkers in other forms of nephrotoxicity. Therefore, the genes included same category with *Kim-1, Fga, Timp1*, and *Slc34a2* could suggest as a novel biomarkers. The results of our gene expression analysis may provide clues for elucidating the mechanism of cephalexin induced nephrotoxicity and evaluating potential biomarkers to assess nephrotoxicity.

Methods

Animals and Chemicals

Experiments were performed approximately 10 weeks old female C57BL/6 (18-23 g) mice (Orient, Korea). The mice were housed in a controlled temperature ($\sim$24°C) and humidity ($\sim$45%) with a 12 hours light/dark cycle. Certified rodent chow (Purina, Korea) and water *ad libitum* were provided except for fasting prior to the day of dissection. Cephalexin was purchased from Sigma Chemical Company. Trizol®

(Molecular research, U.S.A), RNeasy MiniElute Cleanup kit (Qiagen, Germany), Chloroform (Sigma, USA), Isopropanol (Sigma, U.S.A), One-cycle cDNA synthesis kit (Affymetrix Inc., U.S.A), IVT labeling kit (Affymetrix Inc., U.S.A), sample clean-up module (Affymetrix Inc., U.S.A) were used.

Animal Treatment and Sample Preparation

Animals were administered via intraperitoneally (i.p) injection at 10 mL/kg body weight. Cephalexin was dissolved in saline and administered once at dose of 1,000 mg/kg. Control animals were received corresponding quantities (10 mL/kg) of saline. Mice were sacrificed under diethyl ether at 3, 6, and 24 hr after administration. Blood for serum biochemistry evaluation was collected via the inferior vena cava in Vacutainer® (BD, U.S.A) tube. After rapidly removed kidneys, left kidney for genomic study was put into RNAlater® (Qiagen, Germany) and stored at −4°C and right kidney was dipped in 10% neutralized buffered formalin for histopathological examination. RNAlater® was removed after overnight incubation and kidney was stored at −80°C until use.

Blood Biochemistry and Histopathological Analysis

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), blood urea nitrogen (BUN), and creatinine were measured using a DRI-CHEM 3500s (FUJIFILM, Japan). For histopathological analysis, formalin fixed tissues embedded in paraffin, sectioned at 4 μm thickness, and stained with hematoxylin and eosin (H&E) and were examined by light microscope (Nikon E400, Japan) for microscopic examination.

Isolation of RNA

Liver tissues were isolated using Trizol®. In brief, 2 ml homogenized tissue with Trizol® mixed with 0.4 ml chloroform. Following centrifugation, aqueous phase collected and mixed with isopropanaol for precipitation. Isolated total RNA was purified using RNeasy minikit according to the manufacturer's protocol. The total RNA quantified using NanoDrop® ND-1000 (NanoDrop, U.S.A) and RNA integrity were determined by Agilent Bioanalyzer 2100 (Agilent Technologies, U.S.A).

Microarray Analysis

Affymetrix GeneChip® Mouse 430A 2.0 containing over 22,600 probe sets (representing over 14,500 well -substantiated mouse genes) was used for microarray experiment. Total RNA samples were diluted 5 μg/8 μL in RNase-free water. cDNA synthesis, sample labeling, microarray hybridization, washing, and scanning were performed according to the manufacturer's protocols (Affymetrix, Inc., U.S.A) as described previously[18]. The arrays were scanned GeneChip scanner 3000 (Affymetrix, Inc., U.S.A). The preprocessing procedure of resultant cell intensity files (CEL) and following microarray analysis were performed using GenePlex software version 1.8 (Istech Inc., Korea). Data normalization was performed using global scale normalization. The differentially expressed genes were selected based on statistical significance assigned at a minimum 5-fold change at least one time point and Welch's t test. $p<0.01$ were considered as statistically significant. The selected genes were analyzed by two-dimensional hierarchical clustering based on Pearson correlation and Complete Linkage and discrepancies between control and 3, 6, and 24 hr group were visualized by PCA. The classification of pathway for interesting genes was performed using KEGG pathway database. The selected genes were annotated based on NetAffx, linked at http://www.affymetrix.com.

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