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Gene Expression Profiling of Early Renal Toxicity Induced by Gentamicin in Mice

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Abstract

To elucidate the molecular mechanisms associated with early renal injury induced by gentamicin, the most commonly used antibiotics worldwide in the treatment of Gram-negative bacterial infections. We have identified genes differentially expressed at different duration of gentamicin administration. C57BL/6 female mice were treated daily with gentamicin (20 mg/kg, 100 mg/kg, and 200mg/kg) for 7 days and then sacrificed at day 1, 3, and 7 after administration. Standard blood biochemistry and histopathological observation indicative of nephrotoxicity were made. Total RNA was extracted from the kidney for microarray analysis using Affymetrix GeneChip®. Five hundred and seventy eight genes were identified as being either up- or down-regulated over 2-fold changes during early renal injury ($p<0.05$) and were analyzed by hierarchical clustering. The results showed that the genes involved in early immune responses were differentially regulated during early renal injury. Principal component analysis (PCA) confirmed sample separation according to the degree of renal toxicity. In addition, we identified two potential biomarkers that may predict early renal toxicity. This data may contribute to elucidate of the genetic events during early renal injury and to discover the potential biomarkers for nephrotoxicity induced by gentamicin.

Keywords: Gene expression profiling, Toxicogenomics, Gentamicin, Nephrotoxicity

Gentamicin is an aminoglycoside antibiotic that can treat many different types of bacterial infections, particularly Gram-negative infection. However, the high concentration of gentamicin induces the nephrotoxicity acting as the dose-limiting factor in the use of gentamicin[1]. Gentamicin mediates proximal tubule injury in the kidney by inhibiting the lysosomal function of proximal tubular cells and producing phospholipidosis and tubular degeneration[2]. To date, many analyses have been made to understand the mechanism of nephrotoxicity induced by gentamicin using conventional *in vitro* and *in vivo* techniques[3,4], however, an understanding of underlying molecular mechanisms of its nephrotoxicity is still desired.

Recently, extensive efforts for analyzing the entire set of genes involved in toxicity have triggered investigations in microarray-based gene profiling. It has been applied to identify compound-specific and toxicity-specific gene changes in both liver[5-7] and kidney[8-10]. Moreover, recent investigations have demonstrated a gene set which is modulated *in vivo* in response to injury caused by a diverse group of model nephrotoxicants, including cisplatin[8,11], puromycin[11], gentamicin[11], and amphotericin[12]. Although gene expression profiles induced by several nephrotoxins have been studied, the information of gene set related to the early response of renal injury is still limited. Identifying the early responding gene set for toxicity could be useful in developing new drug candidates as well as to understand the molecular mechanisms of renal injury. Especially, discovering the early biomarker for the toxicity of drug candidate in the drug discovery process permits to save time and money. Conventional blood markers such as creatinine and blood urea nitrogen (BUN) have been found insensitive and nonspecific for the detection of renal injury[13].

The toxicogenomic approach should help not only to discover new biomarkers of toxicity but also to explain cellular mechanism of toxicity. In this report, we describe the global gene expression patterns of early renal injury induced by gentamicin in mice model so as to understand the early mechanism and to identify the early biomarkers for nephrotoxicity. Using Affymetrix GeneChip® system, the gene expression profile at different durations after gentamicin administration has been analyzed. Genes that are differentially expressed according to the degree of renal injury were further identified using hierarchical clustering and principal component analysis. In brief, we report on the identification of get set that are dif-

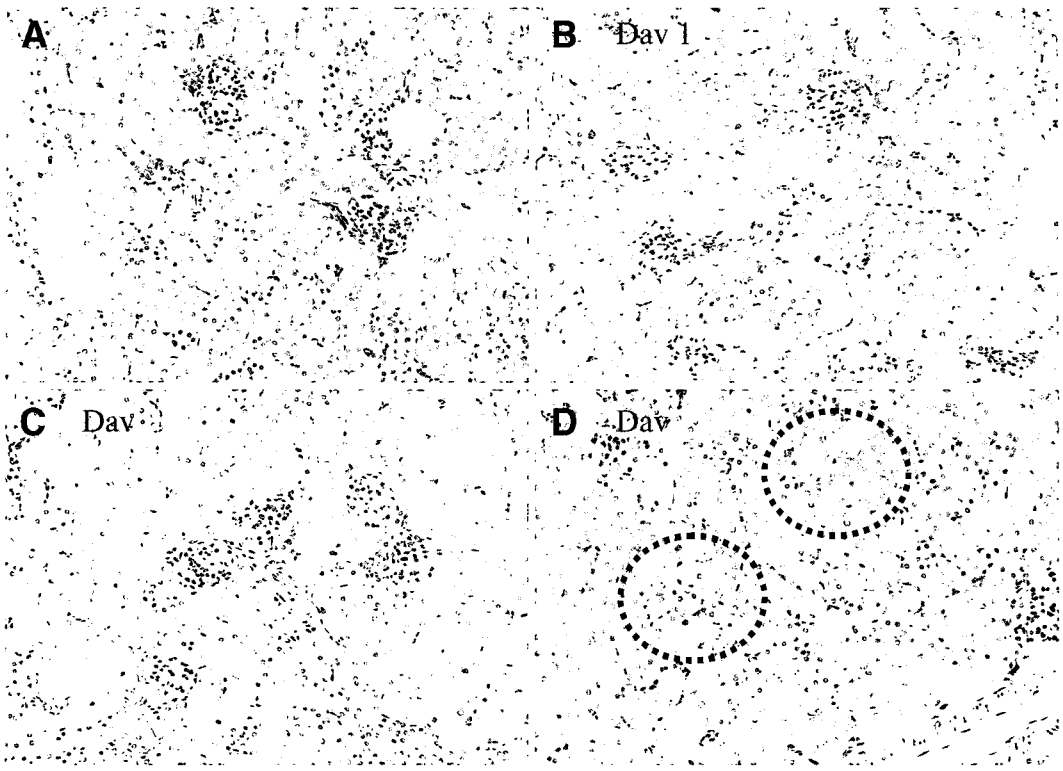

Fig. 1. Light micrograph of kidney from mice administered with 200 mg/kg of gentamicin for day 1, 3, and 7. (A) Control, (B) day 1 treatment, (C) day 3 treatment, and (D) day 7 treatment. The dotted circles indicate the renal injury, tubular basophilia.

ferentially expressed in early renal injury induced by gentamicin and suggest the potential biomarkers for its toxicity in mice.

Blood Biochemistry and Histopathology

The blood biochemistry data for all doses of gentamicin on day 1, 3, and 7 were observed to detect the renal injury. A slight increase in blood urea nitrogen (BUN) was generally observed at the selected doses and duration of administration (data not shown). The high dose caused 1.3-fold increases at day 1 and 3, and 1.2-fold increases at day 7. In the case of low dose administration, higher increase was shown on day 1 compared to day 3 and 7. This result showed that the BUN level barely showed the time-matched and dose-dependant changes during experiment. In the case of creatinine analysis, no significant change was observed at selected doses and any of the time points in this study (data not shown).

Histopathological analysis showed that the low dose administration of gentamicin exhibited no discernable renal pathology at any of the time points but slight tubular basophilia was observed on day 7 after middle dose administration of gentamicin. The mice treated with the highest dose (200 mg/kg) of gentamicin also exhibited renal lesions at day 7 treatments. On day 7, mice in the high dose group showed moderate tubular basophilia, protein casts, and tubular dilation (Fig. 1).

Microarray Analysis

To identify the genes associated with gentamicin induced nephrotoxicity, gene expression profiling from individual kidneys treated with vehicle or gentamicin of high dose level (200 mg/kg) at three time points were analyzed using the Affymetrix GeneChip®. The analysis of samples was performed according to the instructions of the manufacturer, as described in the Methods section.

The differentially expressed genes were compared among 1, 3 and 7 days treatments and time-matched controls. Each data set consisted of an expression array from each of three animals. In all the cases, 578 genes were expressed with over 2-fold modulation ($p < 0.05$, two-tailed, unpaired Welch's t test). These deregulated 578 genes were analyzed using hierarchical clustering (Fig. 2). Hierarchical clustering showed that samples were clustered in dose and time dependent manner. Duration of treatment was respectively differentiated into subclusters. However, the

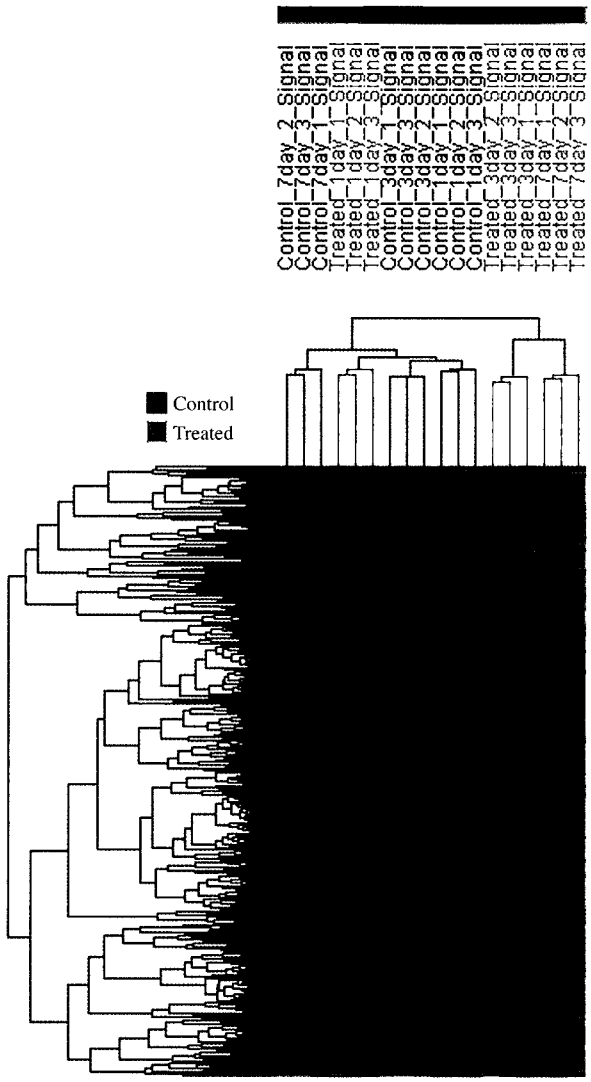

Fig. 2. Hierarchical clustering of renal gene expression profiles after gentamicin administration. Five hundred and seventy eight genes were selected based on statistical significance as described in the Methods section and hierarchical clustering was performed. The colored branch at the top indicates sample group: control (blue) and treated group (pink). The right-handed bar indicates the distinguished categories: down-regulated at day 3 and 7 (white), up-regulated only at day 7 (gray), up-regulated at day 3 and 7 (black), up-regulated at day 1 and 3, but down-regulated at day 7 (dashed) and slightly up regulated at day 7 controls and other treatments.

treated group on day 1 was clustered with the control group, which was branched off separately from other treated groups. The histopathological analysis for these groups revealed that the sample on day 1 and day 3 had no severe renal injury. Overall, samples were clustered according to time dependant toxicity, even though blood biochemistry and histopathological analysis could not distinguish the renal toxicity among the tested group.

Mechanism of Early Renal Injury Induced by Gentamicin

The hierarchical clustering could generally distinguish the five gene expression categories as follows (Fig. 2): down-regulated at day 3 and 7 treatments, up-regulated only at day 7 treatments, up-regulated at day 3 and 7 treatments, up-regulated at day 1 and 3 treatments but down-regulated at day 7 treatments and slightly up-regulated at day 7 of controls. To analyze the molecular mechanism of genes that are differentially expressed in time dependent manner, the KEGG pathway was analyzed. As shown in Fig. 3, up-regulated genes at day 3 and 7 treatments were related to early immune response such as Toll-like receptor (TLR) signaling, and Jak-STAT and MAPK signaling pathway. Toll-like receptors are type I transmembrane proteins that recognize pathogens and activate immune cell responses as a key part of the innate immune system[14]. Jak-STAT & MAPK signaling triggered the downstream of immune response. In addition, many of up-regulated genes only at day 7 treatments were related to downstream of immune response following TLR signaling pathway such as antigen processing and presentation, natural killer cell mediated cytotoxicity and type I diabetes mellitus. Many of genes related to MHC class were included in these pathways. These results suggested that gene set related to immune response was triggered in early renal injury induced by gentamicin, and then apoptosis and necrosis of nephron could follow through sequential signaling pathways.

Differentially Expressed Genes in Gentamicin Induced Nephrotoxicity

To represent the discrepancies of time dependant renal toxicity using principal components analysis, candidate gene set was reduced based on over 2-fold changes ($p < 0.01$) between treated groups and time-matched controls. Totally, 284 differentially regulated genes were selected that could distinguish the time dependant renal toxicity as demonstrated by PCA (Fig. 4). This approach offers an opportunity to visualize expression patterns that can reflect similarities in biological responses. As shown in Fig. 4, three major discrepancies between three treated groups and controls were observed. The treated group on day 1, where no histopathological finding of renal injury was observed, would rather be arranged with control group. The treated group on day 3 was clearly distinguished from that on day 7.

To analyze the gene set associated with renal injury,

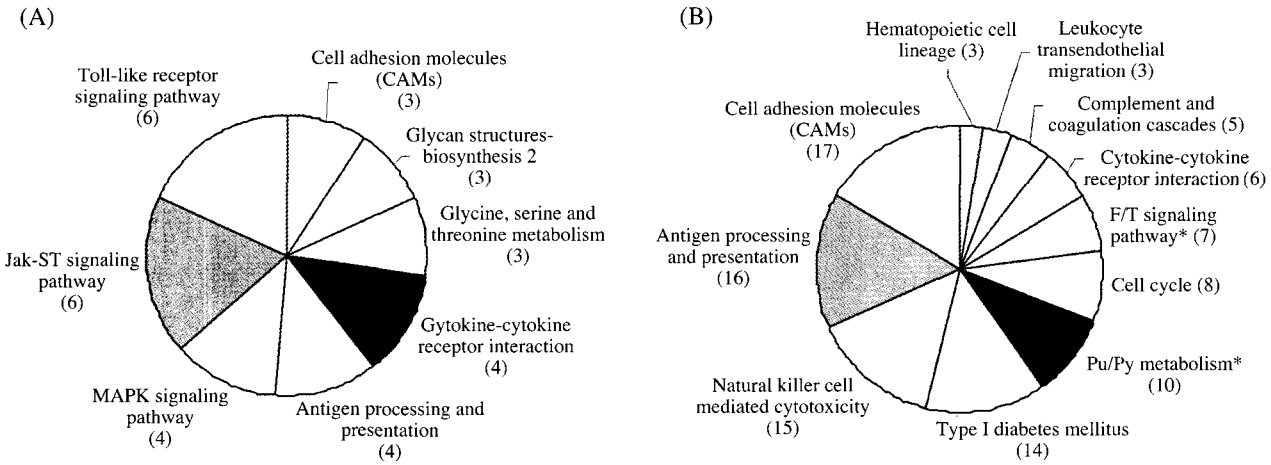

Fig. 3. The pathway analysis of differentially expressed genes using KEGG pathway database. (A) Up-regulated genes at day 3, and 7 treatments (B) Up-regulated genes at only at day 7 treatments. The number of genes assigned to the pathway was represented in parentheses. The long pathway term was abbreviated as follows: F/T signaling pathway*, Fc epsilon RI and T cell receptor signaling pathway and Pu/Py metabolism*, Purine and pyrimidine metabolism.

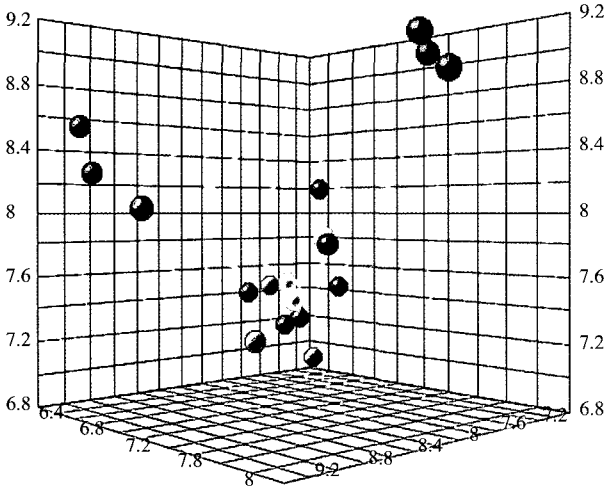

Fig. 4. Principal component analysis for 284 differentially expressed genes. The colored circles represent tested groups as follows: day 1 control, yellow; day 3 control, blue; day 7 control, orange; day 1 treatments, light green; day 3 treatments, purple; day 7 treatments, dark green.

we selected a total of 40 genes, 20 genes for up-regulation and 20 genes for down-regulation, based on significance analysis of over 2-fold changes ($p < 0.05$) that showed significant expression changes at day 7 treatments comparing to time-matched controls. As shown in Table 1, the up-regulated genes at day 7 treatments included known genes involved in immune /inflammatory response (*Ms4a4b, Ms4a4c, Itk, Mx1, 5830443L24Rik, Ccl5, Cxcl9, Ifit1, Cd83*, and *Ccl12*), regeneration/repair (*Havcr1, Uhrf1*, and *Trip13*), metabolism (*Ttk*), and cytoskeleton/cell morphology (*Coro1a*) and also included functionally unknown genes (*D11Lgp2e, LOC677168, 2310016F22Rik*, and *D2Ertd750e*). Genes representing down-regulated expression included those involved in transporter (*Tap1, Slc22a7*, and *Slc25a25*), immune response (*Ecrg*), cell cycle (*Ptk6* and *Gadd45g*), intermediary metabolism (*Sez6l2, Hdc, Klk1b22, Dlx2, Hmgcs2*, and *Tyr*), and cytoskeleton (*Myh1* and *A2m*), and functionally unknown genes (*Chrdl1, Polr3e, C76533, MGC41410*, and *AV344025*), as shown in Table 2. Table 3 shows the gene list for early responding genes when renal damage was initiated. *Cxcl10* and *Gdf15* containing chemokine activity was up-regulated over 2-fold change at all periods of treatment. Up-regulation of these genes was occurred even on day 1 in the absence of extensive histopathological findings.

Discussion

Microarray technologies help in the analysis of expression of a large number of genes for multiple samples at the same time. The application of microarray analysis in toxicology, drug development and drug safety provides an opportunity to improve the pharmaceutics and therapeutics. For pharmaceutical screening, it is highly desirable to identify predictive and early biomarkers of toxicity of a drug exposure. The biochemical analysis of blood had its limitations as early signal for renal toxicity. In present investigations, we found that histopathological findings

Table 1. Up-regulated gene in the kidney of gentamicin treated mice.

Gene title	Gene symbol	Acc. No.	Fold change		
			1 day	3 day	7 day
Immune/Inflammatory response					
membrane-spanning 4-domains, subfamily A, member 4B	*Ms4a4b*	NM_021718	0.8	0.3	5.3
membrane-spanning 4-domains, subfamily A, member 4C	*Ms4a4c*	NM_029499	−0.3	1.4	4.1
IL2-inducible T-cell kinase	*Itk*	NM_010583	0.1	−0.3	4.0
myxovirus (influenza virus) resistance 1	*Mx1*	NM_010846	1.1	1.1	3.7
RIKEN cDNA 5830443L24 gene	*5830443L24Rik*	NM_029509	0.0	0.4	3.5
chemokine (C-C motif) ligand 5	*Ccl5*	NM_013653	0.2	2.0	3.3
chemokine (C-X-C motif) ligand 9	*Cxcl9*	NM_008599	0.0	0.4	3.3
interferon-induced protein with tetratricopeptide repeats 1	*Ifit1*	NM_008331	0.5	2.7	3.2
CD83 antigen	*Cd83*	NM_009856	−0.7	0.4	3.2
chemokine (C-C motif) ligand 12	*Ccl12*	NM_011331	−1.4	0.5	2.9
Regeneration/repair					
hepatitis A virus cellular receptor 1 (Kim1)	*Havcr1*	NM_134248	−1.6	4.0	5.1
ubiquitin-like, containing PHD and RING finger domains, 1	*Uhrf1*	NM_010931	−0.8	0.3	3.3
thyroid hormone receptor interactor 13	*Trip13*	NM_027182	1.6	1.0	3.2
Metabolism					
Ttk protein kinase	*Ttk*	NM_009445	−1.8	0.7	3.0
Cytoskeleton/Cell morphology					
coronin, actin binding protein 1A	*Coro1a*	NM_009898	−0.2	0.5	3.0
Unknown					
DNA segment, Chr 11, Lothar Hennighausen 2, expressed	*D11Lgp2e*	NM_030150	0.3	2.7	5.2
DNA segment, Chr 11, Lothar Hennighausen 2, expressed	*D11Lgp2e*	NM_030150	−2.0	0.5	3.5
hypothetical protein LOC677168	*LOC677168*		0.6	2.6	3.7
cDNA sequence BC020489;RIKEN cDNA 2310016F22 gene	*2310016F22Rik*	XM_001005082	−1.5	1.5	3.5
DNA segment, Chr 2, ERATO Doi 750, expressed	*D2Ertd750e*	NM_026412	−0.5	−1.7	3.0

Up-regulated genes were selected based on ≥2-fold changes on day 7 ($p<0.05$, Welch's t test). The relative fold change of treated group was calculated against each time-matched control and presented as log base 2.

Table 2. Down-regulated genes in the kidney of gentamicin treated mice.

Gene title	Gene symbol	Acc. No.	Fold change		
			1 day	3 day	7 day
Transporter					
transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	*Tap1*	NM_013683	0.4	1.5	−3.9
solute carrier family 22, member 7	*Slc22a7*	NM_144856	0.1	−1.3	−1.8
solute carrier family 25 member 25	*Slc25a25*	NM_146118	0.7	0.5	−1.6
Immune response					
T-cell receptor gamma chain	*Tcrg*		−0.7	0.3	−4.0
Cell cycle					
PTK6 protein tyrosine kinase 6	*Ptk6*	NM_009184	−1.1	0.5	−3.7
growth arrest and DNA-damage-inducible 45 gamma	*Gadd45g*	NM_011817	−0.3	−0.2	−1.7
Intermediary metabolism					
seizure related 6 homolog like 2	*Sez6l2*	NM_144926	−2.3	−0.1	−2.4
histidine decarboxylase	*Hdc*	NM_008230	−1.0	−2.9	−2.4
kallikrein 1-related peptidase b22	*Klk1b22*	NM_010114	−0.3	−2.1	−2.3
histidine decarboxylase	*Hdc*	NM_008230	−0.8	−3.5	−2.2
distal-less homeobox 2	*Dlx2*	NM_010054	−0.2	−0.1	−2.1
3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	*Hmgcs2*	NM_008256	−0.4	0.0	−1.8
tyrosinase	*Tyr*	NM_011661	−0.4	0.8	−1.8
Cytoseleton					
myosin, heavy polypeptide 1, skeletal muscle, adult	*Myh1*	NM_030679	0.2	−1.1	−2.1
alpha-2-macroglobulin	*A2m*	NM_175628	0.8	−1.5	−1.6
Unknown					
chordin-like 1	*Chrdl1*	NM_031258	0.6	−0.8	−3.2
polymerase (RNA) III (DNA directed) polypeptide E	*Polr3e*	NM_025298	−0.5	−0.6	−2.0
expressed sequence C76533	*C76533*		−0.4	−0.4	−1.8
Similar to RIKEN cDNA B430319H21 gene	*MGC41410*		−0.7	−1.1	−1.7
expressed sequence AV344025	*AV344025*		−0.1	0.2	−1.7

Down-regulated genes were selected based on <2-fold changes on day 7 ($p<0.05$, Welch's t test). The relative fold change of treated group was calculated against each time-matched control and presented as log base 2.

Table 3. Selected potential biomarkers for gentamicin induced nephrotoxicity.

Gene title	Gene symbol	Acc. No.	GO term	Fold change		
				1 day	3 day	7 day
Chemokine activity chemokine (C-X-C motif) ligand 10	*Cxcl10*	NM_021274	Chemokine activity	1.5	3.6	2.8
growth differentiation factor 15	*Gdf15*	NM_011819	Chemokine activity	2.8	2.0	1.8

The relative fold change of treated group was calculated against each time-matched control and presented as log base 2. The GO term of assigned gene was represented against molecular process.

were inconsistent with the blood biochemistry. The BUN and creatinine values were found to be insensitive to detect any renal damage although these conventional methods have been widely used in assessing the renal toxicity. The significant changes in BUN and creatinine level are not detectable until the severe damage of the kidney was occurred. Gene expression change occurs early following exposure and before the onset of histopathologic change. Therefore, the genomic biomarkers are more efficiently predictive for detecting the toxicity.

Gene expression profiles during early renal injury provided the information of genes that are initially affected and regulated after renal damage. The histopathological observation at day 7 treatments revealed slight impairment of the kidney such as tubular basophilia. It is likely that the expression pattern of genes that up-regulated at day 7 treatments reflects the biochemical pathway of injury such as inflammation /immune response and regeneration/repair. Up-regulation of genes that are involved in apoptosis, oxidative stress, and necrosis, as shown in severe renal injury induced by several nephrotoxins, was not observed. In the case of down-regulated genes, major class of genes was associated with metabolism and transporters. The transporters involved in the absorption or excretion of xenobiotics or endogenous compounds in the kidney. Down-regulation of genes for transporters might have resulted due to loss of tubular epithelial cells during renal injury.

Among the selected 20 up-regulated genes, kidney injury molecule (*Kim 1*), which has been reported as early biomarker on nephrotoxicity[15], was over-expressed at day 3 and 7 treatments. Other candidate biomarkers such as clusterin (*Clu*)[15], tissue inhibitor of metalloproteinase 1 (*Timp1*)[15], and lipocalin (*Lcn2*)[12] were also up-regulated over 2-fold changes at day 3 and 7 treatments, although they were not selected as highly up-regulated genes (data not shown). Among the 20 up-regulated genes, several genes related to inflammation such as *Ms4a4c, Ccl5, Cscl9*, and functionally unknown genes such as *D11Lpg2e* and *LOC677168* were early over-expressed at day 3 treatments, as well as day 7 treatments. The gene expression patterns of these genes were similar to that of *Kim 1*, early biomarker. These results suggest that these regulated genes may serve as early and sensitive biomarker for gentamicin induced nephrotoxicity. Interestingly, two regulated genes for early renal injury, *Cxcl10* and *Gdf15*, were identified in this experiment and these genes over-expressed at early time point where histopathological finding were not observed yet. *Cxcl10* participates in early inflammatory response and has a hepato-regenerative effect during acute liver injury[16], although it has not been reported that *Cxcl10* is related to renal injury. *Gdf15* is known to be an early mediator of the injury response in liver and lung as well as kidney[17]. The induction of this gene was suggested as a hallmark of immediate-early gene regulation after the organ injury.

Using the microarray analysis, we examined the difference in gene expression profiles in gentamicin induced nephrotoxicity. Our data showed potential biomarkers which are early mediated after renal damage induced by gentamicin, and our additional work is necessary to validate the potential early biomarkers. The identified genes offer valuable information related to molecular mechanisms gentamicin induced nephrotoxicity.

Methods

Animal Treatment

Female C57BL/6 (25-27 g) mice, approximately 10-week-old, were kept in a controlled temperature and humidity with a 12-hour light/dark cycle. Animals were acclimated up to 2 weeks prior to gentamicin administration. All animals were randomly assigned into treatment and control groups (3 mice/group/time point). Gentamicin was purchased from Sigma (G4918) and was administered via intraperitoneal injection (i.p.). Gentamicin was dissolved in 0.9% saline solution and administered once daily up to day 7. Gentamicin was dosed at 20 mg/kg (low), 100 mg/kg (middle), and 200 mg/kg (high). Time-matched control animals received corresponding

quantities of the vehicle. Necropsies were then performed on day 1, 3, and 7.

Tissue Collection

At necropsy, each mouse was anesthetized with diethyl ether and the blood was collected for blood biochemistry analysis. The left kidney was placed in 10% neutral buffered formalin for histology, and the right kidney submerged in an appropriate volume of RNAlater® (Qiagen, Germany) for RNA extraction. The kidneys in RNAlater® were kept at 4°C for overnight and then discarded the reagent, and stored at −80°C until the RNA was extracted.

Blood Biochemistry and Histopathology

Blood biochemistry was estimated after gentamicin administration on days 1, 3, and 7. The blood urea nitrogen (BUN) and creatinine were measured using Fuji Automated Clinical Chemistry Analyzer (Fujifilm, Japan). Average value was presented for blood biochemistry and statistical significance was calculated using two-tailed, unpaired *t* test for comparison between two groups. For histopathology, formalin-fixed renal tissues were embedded in paraffin, cut into 4-μm sections, and were stained with hematoxylin and eosin (H&E), and were analyzed by light microscopy.

RNA Extraction

Kidney samples were homogenized in Trizol reagent (Molecular Research Center, Inc., U.S.A.) and total RNA was isolated using Trizol reagent and purified using RNeasy mini kit (Qiagen, Germany) according to manufacturer instructions. Total RNA was quantified using NanoDrop® (NanoDrop, U.S.A) and the quality of RNA was evaluated using 2100 Bioanalyzer (Agilent Technologies, U.S.A.).

Microarray Analysis

Affymetrix GeneChip® (Mouse Genome 430A 2.0 arrays (containing 22,600 transcripts)) was used for microarray experiment. 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 preprocessing procedure of resultant cell intensity files (CEL) and following microarray analysis were performed using GenePlex software (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 2-fold change and Welch's *t* test ($p<0.05$). The selected deregulated genes were analyzed by hierarchical clustering based on Pearson correlation and Complete Linkage. The highly deregulated genes with over 2-fold changes ($p<0.01$) between treated and control group were used to elucidate the discrimination of gene sets using PCA. The classification of pathway for interesting genes was performed using KEGG pathway database[19]. The selected genes were annotated based on NetAffx, linked at http://www.affymetrix.com.

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