

Comprehensive analysis of differential gene expression profiles on diclofenac-induced acute mouse liver injury and recovery

Heekyoung Chung^a, Hyun-Jun Kim^a, Ki-Seok Jang^a, Mingoo Kim^b,
Jungeun Yang^c, Ju Han Kim^b, Yong-Sung Lee^c, Gu Kong^{a,*}

^a Department of Pathology, College of Medicine, Hanyang University, 17 Haengdang-dong,
Seongdong-gu, Seoul 133-791, Republic of Korea

^b Seoul National University Biomedical Informatics (SNUBI), Seoul National
University College of Medicine, Seoul 110-799, Republic of Korea

^c Department of Biochemistry, College of Medicine, Hanyang University, Seoul 133-791, Republic of Korea

Received 20 February 2006; received in revised form 26 May 2006; accepted 29 May 2006

Available online 12 June 2006

Abstract

Microarray analysis of RNA from diclofenac-administered mouse livers was performed to establish a global gene expression profile during injury and recovery stages at two different doses. A single dose of diclofenac at 9.5 mg/kg or 0.95 mg/kg body weight was given orally, and the liver samples were obtained after 6, 24, and 72 h. Histopathologic studies enabled the classification of the diclofenac effect into injury (6, 24 h) and recovery (72 h) stages. By using the Applied Biosystems Mouse Genome Survey Microarray, a total of 7370 out of 33,315 (22.1%) genes were found to be statistically reliable at $p < 0.05$ by two-way ANOVA, and 602 (1.8%) probes at false discovery rate $< 5\%$ by Significance Analysis of Microarray. Among the statistically reliable clones by both analytical methods, 49 genes were differentially expressed with more than a 1.625-fold difference (which equals 0.7 in \log_2 scale) at one or more treatment conditions. Forty genes and two genes were identified as injury- and recovery-specific genes, respectively, showing that most of the transcriptomic changes were seen during the injury stage. Furthermore, multiple genes involved in oxidative stress, eicosanoid synthesis, apoptosis, and ATP synthesis showed variable transcript levels upon acute diclofenac administration. © 2006 Elsevier Ireland Ltd. All rights reserved.

Keywords: Diclofenac; Mouse; Liver; Toxicogenomics; Applied Biosystems Mouse Genome Survey Microarray

1. Introduction

Diclofenac is a frequently prescribed non-steroidal anti-inflammatory drug (NSAID), which has been associated with occasional hepatic toxicity (Helfgott et al., 1990; Iveson et al., 1990; Ouellette et al., 1991; Purcell et al., 1991; Manoukian and Carson, 1996; de Abajo et

al., 2004). Clinical findings suggest drug hypersensitivity reaction and/or direct toxic effect of the drug or its metabolite as the cause for the toxic effect (Schapira et al., 1986; Helfgott et al., 1990; Iveson et al., 1990; Scully et al., 1993). Recent studies using rat liver mitochondria and freshly isolated rat hepatocytes showed that diclofenac decreases hepatic ATP content and induces hepatocyte apoptosis (Bort et al., 1999; Masubuchi et al., 2000). As a possible mechanism, production of cytochrome P450-mediated metabolic activation, uncoupling of oxidative phosphorylation, mitochondrial permeability transition (MPT), and generation of reactive

* Corresponding author. Tel.: +82 2 2290 8251;
fax: +82 2 2295 1091.

E-mail address: kgong@hanyang.ac.kr (G. Kong).

oxygen species have been suggested (Bort et al., 1999; Masubuchi et al., 2000, 2002; Gomez-Lechon et al., 2003). However, studies on the molecular mechanism of diclofenac hepatotoxicity in the human system, including primary cultured hepatocytes or non-metabolizing cell lines, are very limited (Bort et al., 1999; Gomez-Lechon et al., 2003; Katoh et al., 2004), and global changes in the gene expression have not been tested either in the human or animal model systems.

The application of microarray technology provides a powerful tool in identifying and characterizing changes in gene expression associated with toxicity (Skena et al., 1995). Microarray technique has already been successfully used to investigate altered gene expression in many biological processes including inflammatory disease or tumorigenesis (Heller et al., 1997; Amatschek et al., 2004; Stoughton, 2005). In addition, microarrays can provide highly sensitive and informative markers for toxicity and new information on mechanisms of action through analysis of the gene expression patterns provoked by toxicants (Nuwaysir et al., 1999). Furthermore, they can also supply information about the biological processes that are occurring in a particular model (Rockett and Hellmann, 2004). By conducting sophisticated analysis of gene expression changes over dose and time, one can begin to identify cohorts of coregulated genes, which in turn facilitates both hypothesis generation and practical demonstration of the molecular mechanisms and pathways underlying a model of interest.

In the current study, we established an injury–recovery model of hepatotoxicity by diclofenac and investigated the global transcriptomic changes during the full chronological stages. In addition, gene expression was examined at a lower dose with minor histologic changes, based on our hypothesis that changes at the gene expression level might precede the pathological changes and the highly sensitive microarray technology would enable us to detect transcriptomic changes. We utilized the Mouse Genome Survey Microarray that contains approximately 34,000 60-mer oligonucleotide probes representing 32,381 curated genes that target 44,498 transcripts and nearly 1000 control probes from Applied Biosystems. Although we have seen coregulation of few genes that belong to certain biological pathways, we present our result as the first attempt to identify the global transcriptomic changes upon diclofenac administration, which may be utilized for either class comparison or class prediction (Golub et al., 1999) in the near future, rather than focusing on identifying diclofenac-specific toxic mechanism(s).

2. Materials and methods

2.1. Animal treatment and sample collection

Random-bred male ICR mice approximately 6 weeks of age were obtained from Japan SLC (Hamamatsu, Japan). Mice were allowed water and standard food ad libitum and maintained in a controlled environment in accordance with the guidelines prepared by the National Academy of Sciences. A total of 45 mice were fasted for 12 h before drug administration. In treatment A (high dose), 15 mice randomly selected from 45 fasted mice were given a single dose of 9.5 mg/kg body weight diclofenac (Sigma–Aldrich, St. Louis, MO) in 0.2 ml of water orally with a sonde needle, and five animals were subsequently killed at each time point of 6, 24, and 72 h after drug treatment. In treatment B (low dose), 15 mice were treated with a 1/10th dose of treatment A and sacrificed as above. In treatment C (vehicle), 15 mice were administered with 0.2 ml of water orally with a sonde needle and killed as above. Five animals were neither fasted nor treated with diclofenac (non-treated). High dose used in the present study was based on the results of preliminary studies as the minimal dose that yielded histopathologic hepatotoxicity at 24 h after drug treatment (data not shown). The route of administration has been adopted from the report of Ware et al. (1998). The time point of 24 h was adopted from the report of Amin and Hamza (2005) as histopathologic effect of diclofenac intoxication on liver is seen at 24 h. The time points of 6 and 72 h were chosen arbitrarily as time points for early injury and recovery, respectively. A cross-section of the left lateral lobe of the liver was collected in 10% neutral buffered formalin for histopathology. The remaining portions of liver were collected in RNase-free tubes, treated with RNAlater (Ambion, Austin, TX) and stored in liquid nitrogen until process for RNA extraction.

2.2. Histopathological analysis

The liver tissues collected in formalin were dehydrated, embedded in paraffin, sectioned at 5 μ m, and stained with hematoxylin and eosin. Histopathologic examinations of the liver sections were conducted by a pathologist and peer-reviewed.

2.3. Total RNA isolation

Frozen tissues were pulverized in liquid nitrogen-cooled mortar and pestle apparatus. The powdered tissue was then processed with Trizol (Invitrogen, Carlsbad, CA) for isolation of total RNA, followed by RNeasy (Qiagen, Valencia, CA) purification. Purified total RNAs were analyzed with Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA).

2.4. Expression profiling on applied biosystems expression array system

The Applied Biosystems Mouse Genome Survey Microarray contains 33,315 probes, representing 32,381 curated genes

targeting 44,498 transcripts. Three animals out of five per each group were selected as representative animals from each group on the basis of biochemical and histopathological review, and total RNA from the selected three animals were further processed. Digoxigenin-UTP labeled cRNA was generated and linearly amplified from 1 µg of total RNA from each animal using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit according to the manufacturer's protocol. Array hybridization (three arrays per treatment condition), chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescence Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer following the manufacturer's protocol. Images were auto-gridded, then spotted and spatially normalized. Chemiluminescent signals were quantified, corrected for background, and the final images and feature data were processed using the Applied Biosystems 1700 Chemiluminescent Microarray Analyzer software v1.1. Data and images were collected through an automated process for each microarray using the 1700 analyzer. A total of 30 arrays were run for the RNA samples from 30 animals for 10 experimental conditions (three biological replicates per condition).

2.5. Microarray data analysis

Assay normalized signals with quality flag <100 were used for the analysis. Filtered values were imputed using KNN imputation algorithm (Trojanskaya et al., 2001). Data were transformed by variance stabilizing normalization method (Huber et al., 2002) followed by quantile-normalization across array (Bolstad et al., 2003). Two-way ANOVA and Significant Analysis of Microarray (SAM), both known as powerful "gene-by-gene" tests (Mutch et al., 2002; Yang et al., 2005), were applied to determine differentially expressed genes. Among the many statistical approaches to identify differentially expressed genes (Pan, 2002), ANOVA type approach presents adaptation of the general linear model and generally try to encompass the different sources of systematic variability (Wolfiner et al., 2001; Kerr et al., 2002; Draghici et al., 2003), while SAM is a non-parametric approach by which addition of a constant to the gene-specific standard error avoids false positives due to under

estimation of the gene variance (Tusher et al., 2001). In addition, we adopted the analysis performed by Walker et al. (2006) who utilized the similar long oligonucleotide microarray from Applied Biosystems and analyzed the microarray results with both ANOVA and SAM with minor modifications.

Genes with different dose and time effects were identified and classified into eight groups by two-way ANOVA. The eight groups include four groups with interaction and four without interaction. Both with- and without-interaction groups consist of following four groups: (1) dose- and time-dependent, (2) time-dependent, (3) dose-dependent and (4) independent groups. When a gene had a significant interaction term under saturated linear model, it was classified into one of the four with-interaction groups according to the significance of its time and dose under saturated linear model. Whereas, when with non-significant interaction term under saturated linear model, the gene was classified into one of the four without-interaction group according to the significance of its time and dose term under additive model. The genes in each group were clustered using self-organizing map (SOM). Rectangular grid was used and the dimension of grid was determined for each cluster to have approximately 25 genes. Significance Analysis of Microarray (SAM: <http://www-stat.stanford.edu/~tibs/SAM>) performs a modified *t*-test to identify genes with significant changes in expression and uses permutations to estimate the false discovery rate (FDR). These statistical analyses were performed using R statistical package (Ihaka and Gentleman, 1996) and default parameters were used when unspecified. Gene ontology (GO) and pathway-based enrichment studies were performed for each cluster (Chung et al., 2004; Chung et al., 2005b). Genes were also subjected to Gene Map Annotator and Pathway Profiler (GenMAPP) analysis (<http://www.genmapp.org>), which enables visualization of gene expression by microarray data on maps representing biological pathways and groupings of genes.

2.6. Reverse transcription (RT)-PCR

Semi-quantitative RT-PCR was performed as previously described (Chung et al., 2005a) with the primers listed in Table 1.

Table 1
Primer sequences used to validate the microarray results by semi-quantitative RT-PCR

Gene	Spot ID	Sense primer	Antisense primer
Abi3	527912	AACGCAACCCTACACTACCG	TGGTGAAGTCCCTGGTCTC
Casp3	561901	GGGCCTGTTGAACTGAAAAA	CCGTCTTTGAATTTCTCCA
Ccng2	397393	CTTGCCCTTATGAAGGTGA	GCGTGGTACAAGTGCAAAAA
Cth	579386	GATTTCATGCTGGCTTTGT	ATGCTGGCTGAGAACCCTT
Fbxl20	331560	AGTCTTCGTGGGTGCTTGG	CTTGAATGCCATCCTTGGTT
Gstm6	912773	ACGGAAGCCAGAGTCTTGA	CCACGTGGCTGTTTAAAGT
Micl	704890	CCTAGCTGCTCTCTGCTGT	TCACAGCTCAATCCACAAGC
Serpina12	464315	ACAGCCACATTTGCTCTTCC	ACCTTCAGGCTTCGATGAGA
Trim30	914782	AAAGTGTGCTCAACCCCAAC	GATCCAGGAAAACCTCCGACA
1700009P13Rik	831829	TGTGAGCTTCAGGTTTGGTG	CAGGCTGGCCTTTAGAGTG
GAPDH		CGGAGTCAACGGATTTGGTCTAT	AGCCTTCTCCATGGTTGGTGAAGAC

3. Results

3.1. Establishment of injury and recovery stages

The histopathological evaluation of the livers of the animals treated with high doses of diclofenac showed ballooning change of hepatocytes at 6 h, hepatocellular necrosis with lymphocytic infiltration at 24 h, and recovery at 72 h after drug administration (Fig. 1). Upon low dose treatment, no histologic change was seen at 6 h, ballooning degeneration of hepatocytes at 24 h, and recovery at 72 h. No histologic change was seen in vehicle-treated animals. Based on such histopathologic evaluation, we classified the diclofenac effect on mice liver into injury (6 and 24 h) and recovery (72 h) stages. However, borderline increases in AST and ALT levels were detected in the serum (data not shown). It may have resulted from the relatively mild damage by the doses used in this study, as only spotty necrosis was observed even with the most severe damage at 24 h with high dose of diclofenac.

3.2. Microarray analysis of diclofenac effect on gene expression

Whole microarray data could be found on the web at http://www.snubi.org/publication/TGRC_DIC. To verify the accuracy of the microarray assays, few genes that gave significantly different expression patterns were chosen, and semi-quantitative RT-PCR was performed with primers listed in Table 1. Most of the semi-quantitative RT-PCR results are in reasonable agreement with the microarray results, especially when the concordance was provided by the percentage of genes that showed expression ratios in the same direction (i.e. either up- or down-regulation) rather than the absolute value. Such judgment rationale with the direction of expression has been employed and reported in the literature (Petersen et al., 2005). When the RT-PCR results of 6 conditions were compared to that of non-treated for each individual gene by the direction of change of gene expression levels, 49 sets out of 60 (6 sets per gene, 10 genes total) showed concordant

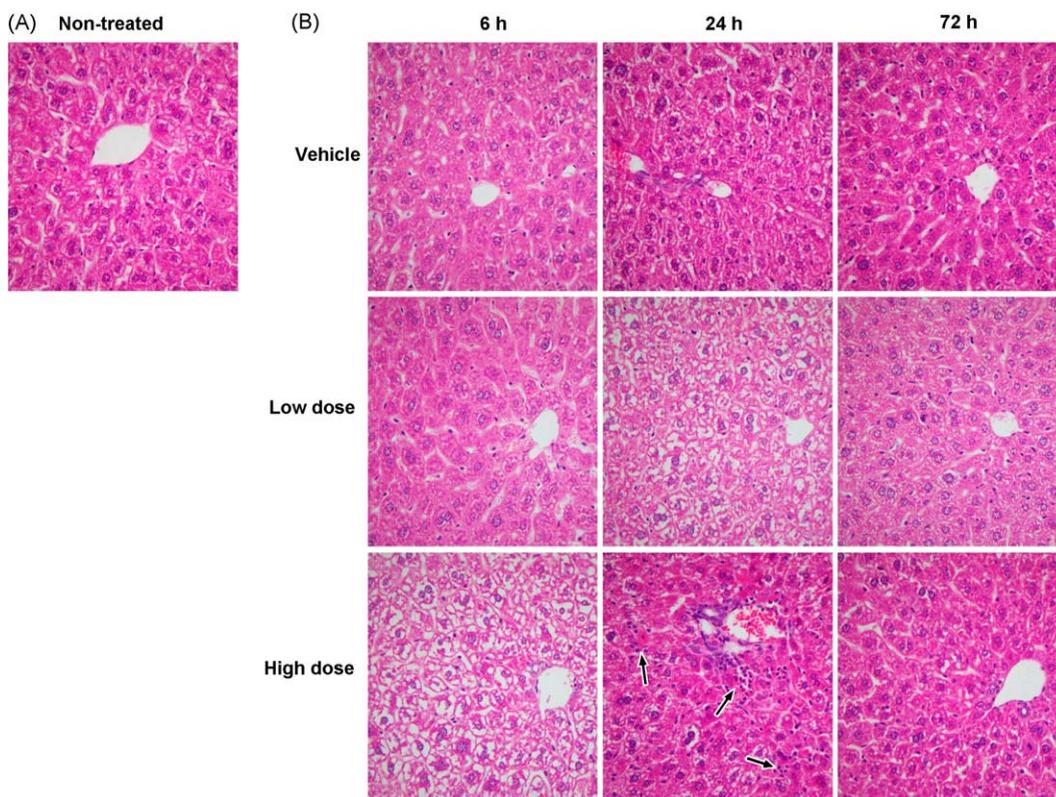


Fig. 1. Sequential histopathologic changes of mouse liver treated with low and high doses of diclofenac (H & E, 400 \times). (A) Normal histology of non-treated liver. (B) Sections from liver treated with vehicle only showed no histologic changes at all three time points. Sections from liver treated with low dose of diclofenac showed no histologic changes at 0 h, ballooning degeneration of hepatocytes at 24 h, and recovery at 72 h. Sections from liver treated with high dose of diclofenac showed ballooning degeneration of hepatocytes at 6 h, spotty hepatocellular necrosis with lymphocytic infiltration at 24 h, and recovery at 72 h.

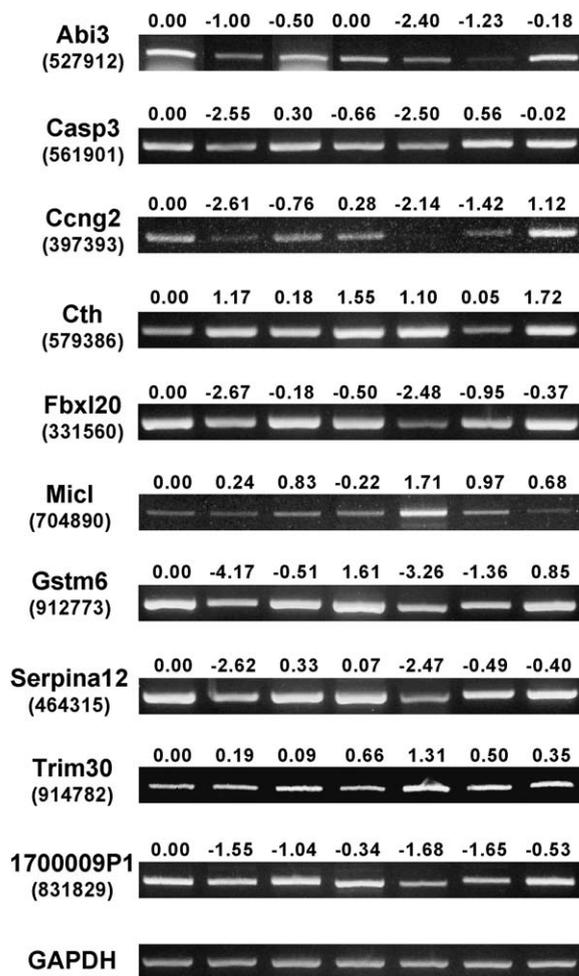


Fig. 2. Semi-quantitative RT-PCR and microarray results of selected clones. Fold changes in \log_2 scale from microarray analysis were illustrated above the semi-quantitative RT-PCR results. Each probe was presented by its gene symbol (left to the panel) and the corresponding spot ID numbers were parenthesised.

expression (82%; Fig. 2). Such percentage of agreement is comparable to the reported values of correlation between oligonucleotide microarray analysis and quantitative real-time PCR results; 60% for 15 genes in Liew and Chow (2006) and 87% for 31 genes in Dallas et al. (2005). Analysis results by two-way ANOVA including grouping, SOM, and pathway analysis could be seen on the web at http://www.snubi.org/publication/TGRC_DIC. Upon two-way ANOVA, a total of 7370 out of 33,315 (22.1%) gene probes were found to be statistically reliable at $p < 0.05$ (supplementary Table 1 at http://www.snubi.org/publication/TGRC_DIC). The significant genes were further classified into eight groups under the terms of the saturated linear model (supplementary Table 1 at <http://www.snubi.org/>

[publication/TGRC_DIC](http://www.snubi.org/publication/TGRC_DIC)). Approximately two thirds of the significant genes (5546 out of 7370) showed dose-dependent effects, implying stronger effect of dose than time in our experimental regimen. The median of the three values (in \log_2 scale) at each condition was calculated, and the fold change versus the non-treated sample (NT) was defined as the expression level. Analysis of the transcriptomic data by two-way ANOVA showed 4566 of 7370 (62.0%) probes were below the value of 0.7 (in \log_2 scale) in all three vehicle-treated samples and above 0.7 in induction or suppression in at least one drug-treated condition (supplementary Table 2 at http://www.snubi.org/publication/TGRC_DIC). Alternatively, when the whole data was statistically analyzed by a modified *t*-test analysis, SAM, against the non-treated control at FDR <5%, 602 out of 33,315 (1.8%) probes were found to be statistically reliable (supplementary Table 3 at http://www.snubi.org/publication/TGRC_DIC). By SAM, 58 of 602 (9.6%) probes were below 0.7 in all three vehicle-treated samples and above 0.7 in induction or suppression in at least one drug-treated condition (supplementary Table 4 at http://www.snubi.org/publication/TGRC_DIC). A comparison of the two lists of genes revealed only 49 genes that were concordantly called “differentially regulated” by both analytical methods (supplementary Table 5 at http://www.snubi.org/publication/TGRC_DIC). However, the term of “differential” expression was not limited to those 49 genes but expanded to include 4566 and 58 genes sorted by two-way ANOVA and SAM, respectively, because inferring any functional roles of the 49 differentially expressed genes was too limited as 18 genes out of 49 (37%) do not have any known function. Throughout this study 0.7-fold was set as a threshold value.

3.3. Injury- and recovery-specific genes

As mentioned previously, acute diclofenac effect was classified into two stages of injury and recovery. Differentially regulated genes were further analyzed to identify injury- and recovery-specific genes as outlined in the flowchart (Fig. 3). Among the 49 genes selected by both analytical methods, 40 genes and 2 genes were identified as injury- and recovery-specific genes, respectively (Table 2). Only two genes belonged to both categories. These genes were individually annotated with the gene ontology (GO) terms (<http://www.geneontology.org>), classified by function, and plotted as injury- (40 clones) versus regeneration-specific clones (2 clones) and up- (32 clones) versus down-regulated clones (10 clones) (Fig. 4). Among the

Table 2
Injury- and recovery-specific genes identified by two-way ANOVA and SAM analyses^a

Spot ID	Gene	GenBank	UniGene	L_6 ^b	L_24	L_72	H_6	H_24	H_72	Function
Injury-specific genes										
Down-regulated										
928053	0610010O12Rik	AK002512	Mm.272527	-1.25	0.09	-0.65	-1.45	-0.13	0.01	
378072	010208K18Rik	NM_028095	Mm.28631	-1.13	0.01	-0.32	0.01	-0.04	-0.64	
806549	6330583M11Rik	NM_024465	Mm.112632	-0.98	-0.10	0.04	-0.59	-0.01	-0.13	
530097	A530050D06Rik	BC023353	Mm.77133	-1.30	-0.16	-0.40	-1.41	-0.08	-0.31	
570984	Abcg8	NM_026180	Mm.26581	-0.93	0.27	0.65	-1.65	0.32	-0.13	Transport
527912	Abi3	BC035943	Mm.129228	-1.00	-0.50	0.00	-2.40	-1.23	-0.18	Cell migration
737168	Aplg2	NM_007455	Mm.238879	-1.04	-0.07	-0.49	-1.46	-0.90	-0.51	Transport (protein)
580857	AU040829	NM_175003	Mm.134338	-2.19	-0.12	-0.50	-1.58	-0.41	-0.03	
482840	BC027342	NM_146251	Mm.29046	-1.24	-0.05	0.23	-1.35	0.12	-0.10	
440174	BC061259	NM_198424	Mm.36852	-1.29	-0.40	-0.36	-0.51	-0.26	-0.13	
812600	C730042F17Rik	NM_145517	Mm.386860	-0.82	-0.24	0.13	-1.15	0.14	-0.13	Protein targeting
551096	Cds2	NM_138651	Mm.284503	-1.90	-0.15	-0.59	-0.91	0.36	-0.58	Metabolism (phospholipid)
626244	Cdw92	AK008866	Mm.270088	-0.62	0.05	-0.28	-1.17	0.07	-0.11	Transport (choline)
908786	Eaf2	NM_134111	Mm.386836	-1.32	-0.33	-0.04	-1.61	-0.29	-0.61	Transcription
901543	Hist1h2ao	NM_178185	Mm.342416	-1.30	-0.61	-0.33	-1.32	0.35	-0.03	Chromosome organization and biogenesis
900157	Hmgb3	NM_008253	Mm.336087	-1.37	-0.35	-0.16	-0.92	-0.38	-0.40	Transcription
928204	Hs1bp3	NM_021429	Mm.309954	-1.45	-0.42	-0.34	-0.61	0.01	-0.56	Signal transduction
924677	Leng5	NM_024168	Mm.379100	-0.87	-0.27	-0.24	-0.51	-0.26	-0.28	mRNA processing
631716	Lsm8	NM_133939	Mm.275158	-0.98	-0.18	-0.28	-0.94	0.31	-0.34	mRNA processing
822012	Ltb4r2	NM_020490	Mm.159670	-1.03	-0.29	0.15	-1.74	-0.23	-0.03	Signal transduction
581260	M6prbp1	NM_025836	Mm.311696	-1.06	0.02	-0.47	-0.79	-0.02	-0.68	Transport (carbohydrate)
551010	Pafah1b3	NM_008776	Mm.597	-2.48	0.22	-0.47	-3.14	0.37	-0.24	Metabolism (lipid)
642099	Plxnb3	NM_019587	Mm.380177	-1.13	-0.44	-0.22	-0.70	-0.48	0.09	Signal transduction
587635	Pop4	NM_025390	Mm.22284	-1.57	0.10	-0.04	-0.22	0.01	0.18	tRNA processing
924253	Sh2d3c	NM_013781	Mm.9593	-0.35	-0.13	0.44	-1.23	-0.27	-0.23	Signal transduction
374634	Tec	NM_013689	Mm.319581	-2.15	-0.02	-0.08	-0.74	-0.38	-0.24	Signal transduction
651122	Tusc3	NM_030254	Mm.272722	-1.75	0.07	-0.49	-1.05	-0.07	-0.19	
929523	Txnrd3	NM_153162	Mm.229332	-1.42	0.28	0.65	-0.69	0.32	-0.09	Transport (electron)
774463	Wispl	NM_018865	Mm.10222	-2.26	-0.48	-0.01	-1.36	0.09	0.22	Signal transduction
509871				-2.29	-0.24	0.19	-1.13	0.14	-0.40	
742311				-0.72	0.01	1.26	-1.59	-0.17	0.58	
Up-regulated										
413987	Cutl2	NM_007804	Mm.332661	2.28	0.72	0.60	2.61	1.24	0.60	Transcription
495744	Cyp2c37	NM_010001	Mm.220317	0.85	0.74	-0.54	2.24	-0.18	-0.65	Transport (electron)
309451	Igtp	NM_018738	Mm.33902	1.03	0.12	0.43	1.87	-0.01	0.48	
420519	LOC209387	NM_199146	Mm.277377	0.53	0.61	0.10	1.69	-0.12	0.51	
704890	Micl	NM_177686	Mm.90241	0.24	0.83	-0.22	1.71	0.97	0.68	Immune response
894280	Mphosph6	NM_026758	Mm.181836	0.67	-0.28	-1.03	1.12	0.34	-0.78	Cell cycle
772594	P2ry12	NM_027571	Mm.386801	-0.18	0.18	0.09	0.40	1.62	-0.11	Signal transduction
931440	Prkab1	NM_031869	Mm.200912	1.09	0.56	-0.37	1.22	0.59	-0.14	Metabolism (carbohydrate)
914782	Trim30	NM_009099	Mm.295578	0.19	0.09	0.66	1.31	0.50	0.35	Transcription
Recovery-specific genes										
Down-regulated										
894280	Mphosph6	NM_026758	Mm.181836	0.67	-0.28	-1.03	1.12	0.34	-0.78	Cell cycle
Up-regulated										
742311				-0.72	0.01	1.26	-1.59	-0.17	0.58	

^a Each value represents the fold change (in log₂ value) vs. the non-treated control.

^b L_6, low dose at 6 h vs. non-treated; L_24, low dose at 24 h vs. non-treated; L_72, low dose at 72 h vs. non-treated; H_6, high dose at 6 h vs. non-treated; H_24, high dose at 24 h vs. non-treated; H_72, high dose at 72 h vs. non-treated.

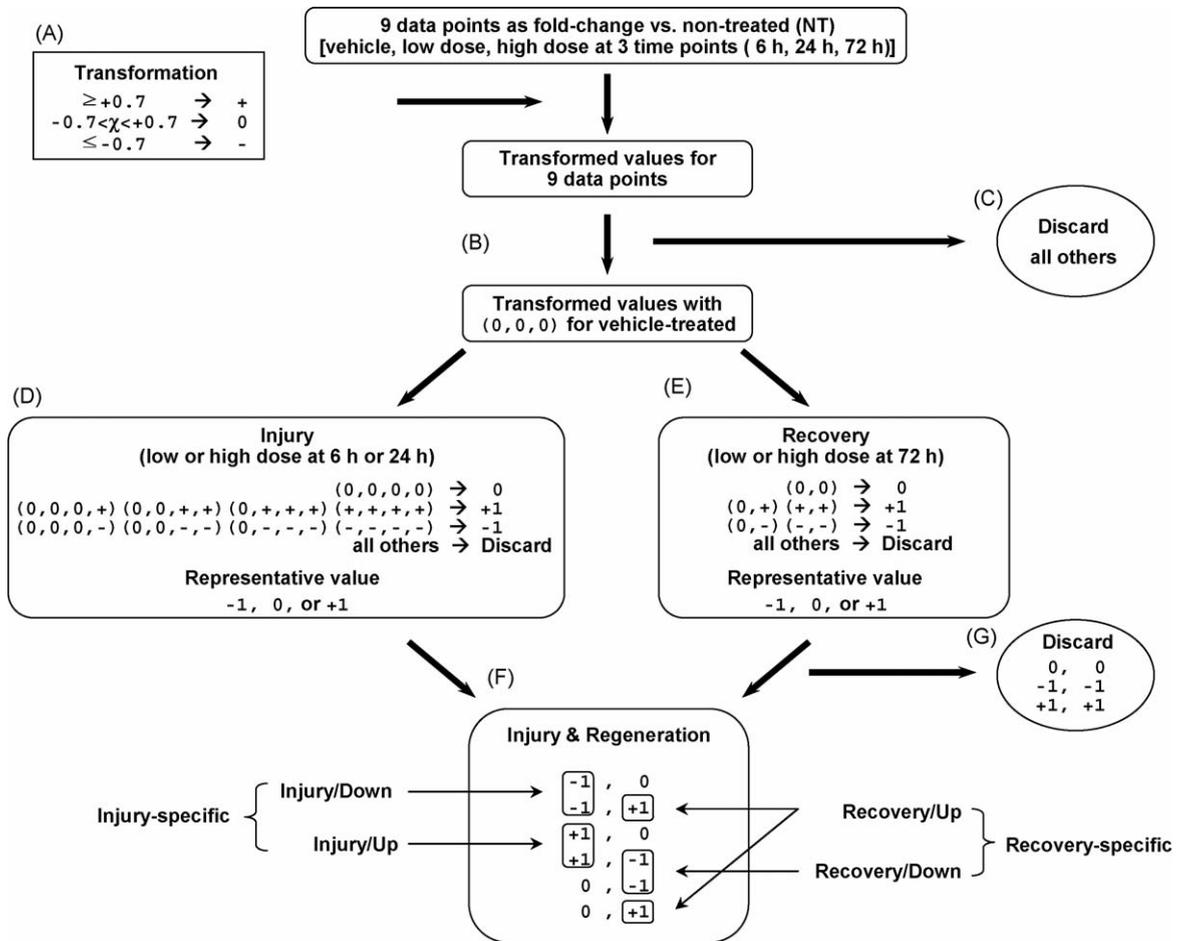


Fig. 3. Flowchart outlining the strategy to define injury- and recovery-specific genes. The numbers used for transformation were fold changes presented as \log_2 values vs. non-treated. The nine numbers per probe were transformed into +, 0, or – according to the rationale depicted in (A). Only the clones with all three vehicle values at 0 (as seen in B) were further analyzed and all other probes were discarded (as shown in C). The resulting transformed values were clustered into injury (as shown in D) and recovery (as seen in E) groups, which had four and two numbers in every individual group. Depending on the combination of the transformed values of each clone, injury and recovery stages were represented with –1, 0, or +1 as shown in (D) and (E), respectively. Injury- and recovery-specific genes were defined as shown in (F). As seen in (F), some of the injury- and recovery-specific genes may overlap. Genes whose expressions were not significantly changed throughout the time course were shown as (0, 0) in (G).

4566 differentially expressed genes isolated by two-way ANOVA, 1355 and 392 probes were classified as injury- and recovery-specific genes, respectively, while 79 genes belonged to both categories (supplementary Table 6 at http://www.snubi.org/publication/TGRC_DIC). These results show that more transcriptomic changes occurred during the injury stage than recovery stage. The differentially expressed genes were further analyzed in the context of different functional categories, which were classified by GenMAPP analysis. Few of those genes that belong to certain functional categories were discussed in detail in the Discussion section. However, due to small number of results concerning the gene expression changes in the human system (Bort et al.,

1999; Gomez-Lechon et al., 2003; Katoh et al., 2004), most of the discussion is limited to those reports which utilized rat or mouse liver tissue or primary hepatocyte cultures.

4. Discussion

4.1. Genes associated with oxidative stress

Although the exact role of oxidative stress in diclofenac hepatotoxicity remains unclear, heme oxygenase-1 (Hmox1), a stress protein, has recently been reported to be induced by the drug (Cantoni et al., 2003). Induction is influenced by changes in the cellular

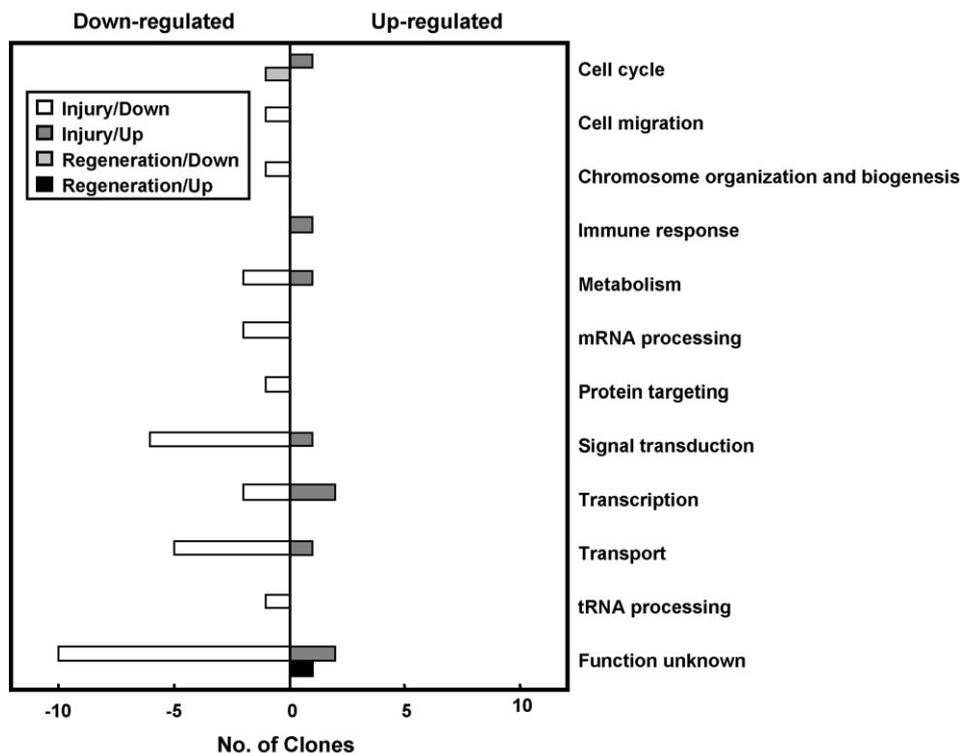


Fig. 4. Injury- and regeneration-specific genes classified by gene function and expression levels.

redox states and by cytochrome P450 activity. Among cytochrome P450, the CYP2C subfamily was reported to be critical because the *in vitro* inhibition of P450-dependent oxidative biotransformation with the selective inhibitor of the CYP2C subfamily markedly reduced the extent of cytotoxicity (Kretz-Rommel and Boelsterli, 1993). In the current study, both Hmox1 and 2 were tested, but neither of them was induced by diclofenac. One possible explanation for such discrepancy may be that the dose and time conditions of Cantoni et al. (2003) are set differently from our experimental setting: the drug has been injected intraperitoneally at 150 mg/kg (compared to 0.95 mg/kg or 9.5 mg/kg orally in our study) and the maximum induction of Hmox1 transcript assessed by Northern analysis is seen at 3 h and subsides completely to normal level at 17 h post-administration (compared to 6, 24, 72 h regimen in our study). Intriguingly, Cyp2c37 (Mm.220317, spot ID 495744), Cyp2c38 (Mm.42100, spot ID 504013) and Cyp2c39 (Mm.42101, spot ID 590638) were all differentially expressed, being induced at the early injury time point (6 h). Such results imply that not only the activity of the CYP2C subfamily is important, but its expression is also augmented by diclofenac. Other differentially expressed genes with oxidoreductase activity include Nqo3a2 (Mm.280230,

spot ID 783824), Txnrd3 (Mm.229332, spot ID 929523), Idh1 (Mm.9925, spot ID 637150), Srd5a1 (Mm.315983, spot ID 456338), and Qprt (Mm.26928, spot ID 924138).

4.2. Genes associated with eicosanoid metabolism

As an NSAID, diclofenac is known to act by potent cyclooxygenase inhibition, reduction of arachidonic acid release, and enhancement of arachidonic acid uptake (Scholer et al., 1986). Although diclofenac is reported as an equipotent inhibitor of Ptgs1 and Ptgs2 (previously known as cyclooxygenase 1 and 2, respectively), no transcriptomic changes were seen for these genes in our study. Instead, expression changes were observed for the genes encoding prostaglandin synthases that catalyze the Ptgs product, prostaglandin H₂ (PGH₂), as substrate: Ptgs (Mm.275434, spot ID 460343) and Pdgd (Mm.143720, spot ID 486733) which encode enzymes that produce prostaglandin I₂ and D₂, respectively, were up-regulated by diclofenac. In addition, transcriptional changes were seen for the Tbxas1 gene (Mm.4054, spot ID 454149), whose gene product converts PGH₂ to thromboxanes A₂. The most notable transcriptomic change was seen with Pla2g12a (Mm.151951, spot ID 751988), which encodes an enzyme with phospholi-

pase A2 activity responsible for arachidonic acid synthesis. Pla2g12a gene expression was highly induced at 6 h in both low and high dose-treated livers. Furthermore, transcript levels of Pla2g4a (Mm.4186, spot ID 498118) and many genes known to indirectly regulate Pla2g4a were also changeable: S100a10 (Mm.1, spot ID 307602), Ednrb (Mm.229532, spot ID 499502), Tnfrsf1a (Mm.1258, spot ID 770146), Anxa1 (Mm.248360, spot ID 915324), Anxa4 (Mm.259702, spot ID 356746), and Anxa5 (Mm.1620, spot ID 689888). Taken together, diclofenac induces transcriptomic changes of many genes involved not only in eicosanoid synthesis but also in its regulation.

4.3. Genes associated with apoptosis

Diclofenac is also known to induce apoptosis in hepatocytes by alteration of mitochondrial function and generation of reactive oxygen species (Gomez-Lechon et al., 2003). GenMAPP analysis revealed that multiple genes involved in the apoptotic pathway were also changeable by diclofenac. In the tumor necrosis factor (TNF)-alpha pathway, Tngsf10 (Mm.1062, spot ID 464424), Tnfrsf1a (Mm.1258, spot ID 770146) and its downstream regulators such as Nfkbib (Mm.220333, spot ID 429553), Nfkb1a (Mm.170515, spot ID 635842), Bok (Mm.3295, spot ID 364104), Trp53 (Mm.222, spot ID 788934), Myc (Mm.2444, spot ID 819312), and Jun (Mm.275071, spot ID 534427) were changed. In the Fas pathway Ripk2 (Mm.112765, spot ID 416043) and its downstream regulators Map3k1 (Mm.15918, spot ID 583197), Lmnb1 (Mm.4105, spot ID 626096 and 629918), Lmnb2 (Mm.7362, spot ID 927483), and Jun (Mm.275071, spot ID 534427) were changed. In particular, expression of many caspase genes was shown to be affected by diclofenac. Casp1 (Mm.1051, spot ID 924714) was seen to be highly induced at 6 h in both low and high doses. Casp11 (Mm.1569, spot ID 419016) was moderately induced with low dose at 72 h. Although the activity of caspase 3 (Casp3; Mm.34405, spot IDs 456269 and 561901) has been reported to be induced by diclofenac in rat hepatocytes in a dose-dependent manner (Gomez-Lechon et al., 2003), transcriptomic analysis showed reduced expression at 6 h in both low and high doses in two separate spots on the microarray chip. Casp6 (Mm.281379, spot ID 804355) was down-regulated with low dose at 6 h. Tnfsf10 was up-regulated in all conditions, but to a less degree at 72 h. Bcl-2-related ovarian killer protein (Bok; Mm.3295, spot ID 364104) was moderately down-regulated with both low and high doses at 6 h. I kappa B alpha (Nfkb1a) was moderately induced at 72 h by both doses, while mitogen-activated

protein kinase kinase kinase 1 (Map3k1) was reduced with high dose at 6 h. Anti-apoptotic Birc1 (Mm.8552, spot ID 510606) was highly reduced with low dose at 24 h, but augmented with high dose at 72 h. Although not limited to apoptotic pathway, Myc was down-regulated at both 24 and 72 h, and Jun was up-regulated with low dose at 6 h and down-regulated with high dose at 24 h. Interestingly, expression of heat shock protein (HSP) genes Hspa1a (Mm.6388, spot ID 591371) and Hspb1 (Mm.13849, spot ID 485972) were also changed: Hspa1a was up-regulated at 72 h and Hspb1 was down-regulated at 24 h regardless of diclofenac doses. Taken together, many genes associated with apoptosis were induced and/or repressed, providing an additional mechanism for induced cell death by diclofenac.

4.4. Genes associated with glycogen and glucose metabolism

Diclofenac is reported to decrease hepatic ATP content (Bort et al., 1999; Masubuchi et al., 2002). Although diclofenac, its hydroxylated metabolites, and mitochondrial permeability transition are reported as mechanisms to reduce ATP levels, we explored the possibility if genes involved in ATP synthesis via the glycogen and glucose metabolic pathways were affected by diclofenac. Gene expression in glycogen metabolism, glycolysis and gluconeogenesis, Krebs cycle, and electron transport chain pathways were examined by GenMAPP analysis. Phosphorylase kinase alpha (Phka) regulatory subunit and phosphorylase kinase gamma (Phkg) catalytic subunits were down-regulated by diclofenac. Although both skeletal (Phka1 and Phkg1)- and liver (Phka2 and Phkg2)-specific isoforms were tested by microarray technique, only the latter isoforms were affected by diclofenac (Phka2; Mm.350712, spot ID 739893 and Phkg2; Mm.274473, spot ID 450816). Likewise, among the glycogen phosphorylases examined, only the liver isoform (Pygl; Mm.256926, spot ID 349572) not the muscle isoform (Pygm; Mm.27806, spot ID 583078), showed expression changes. Such results strongly imply the exquisite accuracy and sensitivity of the microarray analysis performed in this study. Phosphoglucomutase (Pgm1; Mm.2325, spot ID 314328) was down-regulated, whereas protein phosphatase 2A catalytic subunit, alpha isoform (Ppp2ca; Mm.260288, spot ID 473515) and B56 regulatory subunit, epsilon isoform (Ppp2r5e; Mm.259626, spot ID 694902) were up-regulated. All of these gene changes favor glycogen synthesis over degradation, which may serve as a possible mechanism for reduced ATP content by diclofenac. Similarly, many genes associated with glycolysis, Krebs cycle,

electron transport chain were down-regulated: G6pc (Mm.18064, spot ID 333144), Gpi1 (Mm.589, spot ID 662059 and 900795), Tpi1 (Mm.4222, spot ID 359347), Got1 (Mm.19039, spot ID 335092), Ldh2 (Mm.9745, spot ID 924435), Pdha1 (Mm.34775, spot ID 552612), Pdhb (Mm.301527, spot ID 477636 and 870999), Dlat (Mm.285076, spot ID 856498), Pdhx (Mm.315011, spot ID 767843), Sdha (Mm.158231, spot ID 744136), and Cox7a1 (Mm.12907, spot ID 304467). Few up-regulated genes include Fbp2 (Mm.289741, spot ID 538549), Ldh1 (Mm.29324, spot ID 897674 and 903368), Idh3a (Mm.279195, spot ID 922492), and Slc25a14 (Mm.34953, spot ID 301877). Intriguingly, Slc25a14 is located in the mitochondrial inner membrane and known to have a respiration uncoupling activity (Sanchis et al., 1998). Augmented expression of the uncoupling gene may contribute to reduced hepatic ATP content.

In summary, we report the full chronological gene expression profile of mouse liver upon diclofenac administration by histopathology and microarray analyses. In particular, the diclofenac effect was divided into injury and recovery stages, and genes specific to each stage were discussed in association with functional categories. Many genes associated with oxidative stress, eicosanoid metabolism, apoptosis and ATP synthesis yielded differential expression throughout the time course and doses investigated. Comparing our results with those found in humans was limited, as only handful of results is reported for molecular changes in the human system (Bort et al., 1999; Gomez-Lechon et al., 2003; Katoh et al., 2004). Furthermore, it is too early to conclude that the gene expression profiles we observed are exclusively diclofenac-specific. Microarray analyses of various hepatotoxins that belong to the same pharmacological class (e.g. NSAIDs) or cause different patterns of hepatic damages should be performed to identify the “fingerprint” gene expression patterns of diclofenac. However, we believe that our results would be valuable in not only understanding the mechanism of diclofenac-induced liver injury and subsequent recovery, but also for future application such as building a hepatotoxin toxochip.

Acknowledgement

This work was supported by Korea Food and Drug Administration grant (KFDA-05122-TGP-584) to G. Kong.

References

Amatschek, S., Koenig, U., Auer, H., Steinlein, P., Pacher, M., Gruenfelder, A., Dekan, G., Vogl, S., Kubista, E., Heider, K.H., Stratowa,

- C., Schreiber, M., Sommergruber, W., 2004. Tissue-wide expression profiling using cDNA subtraction and microarrays to identify tumor-specific genes. *Cancer Res.* 64 (3), 844–856.
- Amin, A., Hamza, A.A., 2005. Oxidative stress mediates drug-induced hepatotoxicity in rats: a possible role of DNA fragmentation. *Toxicology* 208 (3), 367–375.
- Bolstad, B.M., Irizarry, R.A., Astrand, M., Speed, T.P., 2003. A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics* 19 (2), 185–193.
- Bort, R., Ponsoda, X., Jover, R., Gomez-Lechon, M.J., Castell, J.V., 1999. Diclofenac toxicity to hepatocytes: a role for drug metabolism in cell toxicity. *J. Pharmacol. Exp. Ther.* 288 (1), 65–72.
- Cantoni, L., Valaperta, R., Ponsoda, X., Castell, J.V., Barelli, D., Rizzardini, M., Mangolini, A., Hauri, L., Villa, P., 2003. Induction of hepatic heme oxygenase-1 by diclofenac in rodents: role of oxidative stress and cytochrome P-450 activity. *J. Hepatol.* 38 (6), 776–783.
- Chung, H., Hong, D.-P., Jung, J.-Y., Kim, H.-J., Jang, K.-S., Sheen, Y.-Y., Ahn, J.-I., Lee, Y.-S., Kong, G., 2005a. Comprehensive analysis of differential gene expression profiles on carbon tetrachloride-induced rat liver injury and regeneration. *Toxicol. Appl. Pharmacol.* 206 (1), 27–42.
- Chung, H.J., Kim, M., Park, C.H., Kim, J., Kim, J.H., 2004. ArrayX-Path: mapping and visualizing microarray gene-expression data with integrated biological pathway resources using Scalable Vector Graphics. *Nucl. Acids Res.* 32 (Web Server issue), W460–W464.
- Chung, H.J., Park, C.H., Han, M.R., Lee, S., Ohn, J.H., Kim, J., Kim, J.H., 2005b. ArrayXPath II: mapping and visualizing microarray gene-expression data with biomedical ontologies and integrated biological pathway resources using Scalable Vector Graphics. *Nucl. Acids Res.* 33 (Web Server issue), W621–W626.
- Dallas, P.B., Gottardo, N.G., Firth, M.J., Beesley, A.H., Hoffmann, K., Terry, P.A., Freitas, J.R., Boag, J.M., Cummings, A.J., Kees, U.R., 2005. Gene expression levels assessed by oligonucleotide microarray analysis and quantitative real-time RT-PCR—how well do they correlate? *BMC Genomics* 6 (1), 59.
- de Abajo, F.J., Montero, D., Madurga, M., Garcia Rodriguez, L.A., 2004. Acute and clinically relevant drug-induced liver injury: a population based case-control study. *Br. J. Clin. Pharmacol.* 58 (1), 71–80.
- Draghici, S., Kulaeva, O., Hoff, B., Petrov, A., Shams, S., Tainsky, M.A., 2003. Noise sampling method: an ANOVA approach allowing robust selection of differentially regulated genes measured by DNA microarrays. *Bioinformatics* 19 (11), 1348–1359.
- Golub, T.R., Slonim, D.K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J.P., Coller, H., Loh, M.L., Downing, J.R., Caligiuri, M.A., Bloomfield, C.D., Lander, E.S., 1999. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. *Science* 286 (5439), 531–537.
- Gomez-Lechon, M.J., Ponsoda, X., O'Connor, E., Donato, T., Castell, J.V., Jover, R., 2003. Diclofenac induces apoptosis in hepatocytes by alteration of mitochondrial function and generation of ROS. *Biochem. Pharmacol.* 66 (11), 2155–2167.
- Helfgott, S.M., Sandberg-Cook, J., Zakim, D., Nestler, J., 1990. Diclofenac-associated hepatotoxicity. *JAMA* 264 (20), 2660–2662.
- Heller, R.A., Schena, M., Chai, A., Shalon, D., Bedilion, T., Gilmore, J., Woolley, D.E., Davis, R.W., 1997. Discovery and analysis of inflammatory disease-related genes using cDNA microarrays. *Proc. Natl. Acad. Sci. U.S.A.* 94 (6), 2150–2155.

- Huber, W., von Heydebreck, A., Sultmann, H., Poustka, A., Vingron, M., 2002. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 18 (Suppl. 1), S96–S104.
- Ihaka, R., Gentleman, R., 1996. R: a language for data analysis and graphics. *J. Comput. Graph. Stat.* 5 (3), 299–314.
- Iveson, T.J., Ryley, N.G., Kelly, P.M., Trowell, J.M., McGee, J.O., Chapman, R.W., 1990. Diclofenac associated hepatitis. *J. Hepatol.* 10 (1), 85–89.
- Katoh, M., Matsui, T., Nakajima, M., Tateno, C., Kataoka, M., Soeno, Y., Horie, T., Iwasaki, K., Yoshizato, K., Yokoi, T., 2004. Expression of human cytochromes P450 in chimeric mice with humanized liver. *Drug Metab. Dispos.* 32 (12), 1402–1410.
- Kerr, M., Afshari, C., Bennett, L., Bushel, P., Martinez, J., Walker, N., Churchill, G., 2002. Statistical analysis of a gene expression microarray experiment with replication. *Stat. Sinica* 12, 203–218.
- Kretz-Rommel, A., Boelsterli, U.A., 1993. Diclofenac covalent protein binding is dependent on acyl glucuronide formation and is inversely related to P450-mediated acute cell injury in cultured rat hepatocytes. *Toxicol. Appl. Pharmacol.* 120 (1), 155–161.
- Liew, K.J., Chow, V.T., 2006. Microarray and real-time RT-PCR analyses of a novel set of differentially expressed human genes in ECV304 endothelial-like cells infected with dengue virus type 2. *J. Virol. Meth.* 131 (1), 47–57.
- Manoukian, A.V., Carson, J.L., 1996. Nonsteroidal anti-inflammatory drug-induced hepatic disorders. Incidence and prevention. *Drug Safety* 15 (1), 64–71.
- Masubuchi, Y., Nakayama, S., Horie, T., 2002. Role of mitochondrial permeability transition in diclofenac-induced hepatocyte injury in rats. *Hepatology* 35 (3), 544–551.
- Masubuchi, Y., Yamada, S., Horie, T., 2000. Possible mechanism of hepatocyte injury induced by diphenylamine and its structurally related nonsteroidal anti-inflammatory drugs. *J. Pharmacol. Exp. Ther.* 292 (3), 982–987.
- Mutch, D.M., Berger, A., Mansourian, R., Rytz, A., Roberts, M.A., 2002. The limit fold change model: a practical approach for selecting differentially expressed genes from microarray data. *BMC Bioinform.* 3, 17.
- Nuwaysir, E.F., Bittner, M., Trent, J., Barrett, J.C., Afshari, C.A., 1999. Microarrays and toxicology: the advent of toxicogenomics. *Mol. Carcinogen.* 24 (3), 153–159.
- Ouellette, G.S., Slitzky, B.E., Gates, J.A., Lagarde, S., West, A.B., 1991. Reversible hepatitis associated with diclofenac. *J. Clin. Gastroenterol.* 13 (2), 205–210.
- Pan, W., 2002. A comparative review of statistical methods for discovering differentially expressed genes in replicated microarray experiments. *Bioinformatics* 18 (4), 546–554.
- Petersen, D., Chandramouli, G.V., Geoghegan, J., Hilburn, J., Paarlberg, J., Kim, C.H., Munroe, D., Gangi, L., Han, J., Puri, R., Staudt, L., Weinstein, J., Barrett, J.C., Green, J., Kawasaki, E.S., 2005. Three microarray platforms: an analysis of their concordance in profiling gene expression. *BMC Genomics* 6 (1), 63.
- Purcell, P., Henry, D., Melville, G., 1991. Diclofenac hepatitis. *Gut* 32 (11), 1381–1385.
- Rockett, J.C., Hellmann, G.M., 2004. Confirming microarray data—is it really necessary? *Genomics* 83 (4), 541–549.
- Sanchis, D., Fleury, C., Chomiki, N., Gubern, M., Huang, Q., Neverova, M., Gregoire, F., Easlick, J., Raimbault, S., Levi-Meyruis, C., Miroux, B., Collins, S., Seldin, M., Richard, D., Warden, C., Bouillaud, F., Ricquier, D., 1998. BMCP1, a novel mitochondrial carrier with high expression in the central nervous system of humans and rodents, and respiration uncoupling activity in recombinant yeast. *J. Biol. Chem.* 273 (51), 34611–34615.
- Schaphira, D., Bassan, L., Nahir, A.M., Scharf, Y., 1986. Diclofenac-induced hepatotoxicity. *Postgrad. Med. J.* 62 (723), 63–65.
- Schena, M., Shalon, D., Davis, R.W., Brown, P.O., 1995. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science* 270 (5235), 467–470.
- Scholer, D.W., Ku, E.C., Boettcher, I., Schweizer, A., 1986. Pharmacology of diclofenac sodium. *Am. J. Med.* 80 (4B), 34–38.
- Scully, L.J., Clarke, D., Barr, R.J., 1993. Diclofenac induced hepatitis. 3 cases with features of autoimmune chronic active hepatitis. *Digest. Dis. Sci.* 38 (4), 744–751.
- Stoughton, R.B., 2005. Applications of DNA microarrays in biology. *Annu. Rev. Biochem.* 74, 53–82.
- Troyanskaya, O., Cantor, M., Sherlock, G., Brown, P., Hastie, T., Tibshirani, R., Botstein, D., Altman, R.B., 2001. Missing value estimation methods for DNA microarrays. *Bioinformatics* 17 (6), 520–525.
- Tusher, V.G., Tibshirani, R., Chu, G., 2001. Significance analysis of microarrays applied to the ionizing radiation response. *Proc. Natl. Acad. Sci. U.S.A.* 98 (9), 5116–5121.
- Walker, S.J., Wang, Y., Grant, K.A., Chan, F., Hellmann, G.M., 2006. Long versus short oligonucleotide microarrays for the study of gene expression in nonhuman primates. *J. Neurosci. Meth.* 152 (1/2), 179–189.
- Ware, J.A., Graf, M.L., Martin, B.M., Lustberg, L.R., Pohl, L.R., 1998. Immunochemical detection and identification of protein adducts of diclofenac in the small intestine of rats: possible role in allergic reactions. *Chem. Res. Toxicol.* 11 (3), 164–171.
- Wolfinger, R.D., Gibson, G., Wolfinger, E.D., Bennett, L., Hamadeh, H., Bushel, P., Afshari, C., Paules, R.S., 2001. Assessing gene significance from cDNA microarray expression data via mixed models. *J. Comput. Biol.* 8 (6), 625–637.
- Yang, Y.H., Xiao, Y., Segal, M.R., 2005. Identifying differentially expressed genes from microarray experiments via statistic synthesis. *Bioinformatics* 21 (7), 1084–1093.