Toxicogenomic Study to Identify Potential New Mechanistic Markers on Direct-Acting Mutagens in Human Hepatocytes (THLE-3)

Youn-Jung Kim[1], **Mi-Kyung Song**[1], **Mee-Song**[1] & **Jae-Chun Ryu**[1]

[1]Cellular and Molecular Toxicology Laboratory, Korea Institute of Science & Technology P.O. Box 131, Cheongryang, Seoul 130-650, Korea
Correspondence and requests for materials should be addressed to J. C. Ryu (ryujc@kist.re.kr)

Abstract

Exposure to DNA-damaging agents can elicit a variety of stress-related responses that may alter the expression of genes associated with numerous biological pathways. We used 19 k whole human genome chip to detect gene expression profiles and potential signature genes in human normal hepatocytes (THLE-3) by treatment of five direct acting mutagens, furylfuramide (AF-2), N-nitroso-N-methylurea (MNU), methylmethanesulfonate (MMS), 4-nitroquinoline-N-oxide (4-NQO) and 2-nitrofluorene (2NF) of the IC_{20} concentration for 3 h. Fifty one up-regulated common genes and 45 down-regulated common genes above 1.5-fold by five direct-acting mutagens were identified by clustering analysis. Many of these changed genes have some association with apoptosis, control of cell cycle, regulation of transcription and signal transduction. Genes related to these functions, as TP73L, E2F5, MST016, SOX5, MAFB, LIF, SII3, TFIIS, EMR1, CYTL1, CX3CR1 and RHOH are up-regulated. Down-regulated genes are ALOX15B, xs155, IFITM1, BATF, VAV2, CD79A, DCDC2, TNFSF8 and KOX8. We suggest that gene expression profiling on mutagens by toxicogenomic analysis affords promising opportunities to reveal potential new mechanistic markers of genotoxicity.

Keywords: Mutagen, DNA damage, Toxicogenomics, Gene expression analysis, THLE-3 cell

The major research goals developing biomarkers are the development and validation of biomarkers that permit the prediction of the risk of disease or hazard. Toxicogenomic study has been widely used to characterize toxicological properties of disease or hazard such as mutation[1]. Through previous studies in our laboratory, we confirmed toxicogenomic effects of several hazardous chemicals involved in inducing cancer or adverse effects[1,2]. In this paper, we subjected to mutagens for observing their effects on genes and evaluating the associated mechanisms using toxicogenomic tools.

Furylfuramide (AF-2), N-nitroso-N-methylurea (MNU), methylmethanesulfonate (MMS), 4-nitroquinoline-N-oxide (4-NQO) and 2-nitrofluorene (2NF) are known as a direct mutagen or a potent mutagen. These chemicals cause DNA damage by a variety of mechanisms, and DNA damage triggers a variety of cellular responses including the transcriptional activation of genes regulating DNA repair, cell cycle arrest and apoptosis. AF-2 had been widely used as a food preservative and its genetic effects were reported by many researchers, who led to its classification as a potent mutagen and a directing-acting alkylating agent[3]. MNU, MMS and 4-NQO are also known as alkylating agents[4-7]. 2NF has been detected in particulate emissions from diesel engines, kerosene heaters and gas burners[8]. And the other mutagens are found in surrounding environment. Furthermore, most of these mutagens are reported as carcinogen that induces tumor. Therefore, it is very important to explore the influence of these mutagens which have deadly effects on DNA, so we have been studying about mechanism of mutagens in gene levels.

There are several methods used to study in gene level. Especially, microarray technologies have been widely used for comprehensive gene expression analysis as well as mutation and single nucleotide polymorphism detection[1,2,9-11]. This large-scale microarray analysis of gene expression enables researchers to analyze simultaneous changes in thousands of genes and identify significant patterns.

The aim of this study is the identification of potential gene-based markers on mutagens. We examined global gene expression in a small number of well-matched exposed-control subject pairs. Genes with differential expression were then ranked and selected for further examination using several forms of statistical analysis. The identification of differentially expressed genes (DEGs) may assist in the identification of potential biomarker and may understand molecular

toxicological mechanisms of AF-2, MNU, MMS, 4-NQO and 2NF in human hepatocytes.

Cell Viability of AF-2, MNU, MMS, 4-NQO and 2NF

Relative survivals of THLE-3 cells following exposure to a range of concentrations of AF-2, MNU, MMS, 4-NQO and 2NF were determined by MTT assay. The survival percentage relative to solvent control (DMSO or DW) was determined as a percentage of OD value measured after treatment. Based on the results of MTT assay, 20% inhibitory concentration (IC_{20}) of each compound was calculated. AF-2, MNU, MMS, 4-NQO and 2NF reduced cell viability gradually at increasing concentrations (data not shown). The IC_{20} value for AF-2, MNU, MMS, 4-NQO and 2NF are 243.12 μM, 2,093.97 μM, 510.37 μM, 0.83 μM and 80.78 μM, respectively.

Gene Expression Altered by Direct Mutagens, AF-2, MNU, MMS, 4-NQO and 2NF

THLE-3 cells were treated with 243.12 μM AF-2, 2,093.97 μM MNU, 510.37 μM MMS, 0.83 μM 4-NQO and 80.78 μM 2NF, respectively, for 3 hrs, and their RNA were subjected to microarray analysis. For each treatment, genes with statistically significant expression changes were identified ($P < 0.05$). Five independent experimental samples for each treatment group were analyzed to determine RNA transcript levels. Only those genes, which displayed either greater than or equal to a 1.5 fold up- and down-regulation, have been considered for this study. Hierarchical clustering was used to aid in visualization and biological interpretation of this extensive data set, and in particular, to identify correlated expression patterns. Hierarchical clustering was applied across the five compounds, using a combined list of genes identified to be altered statistically significant in at least one of the sample studied relative to control (Figure 1). From the clustering analysis, we can assume that the gene expression profiles of MMS and 2NF are similar and 4-NQO, MNU and AF-2 are similar. Oligonucleotide microarray analysis was employed to characterize the cells response to AF-2, MNU, MMS, 4-NQO and 2NF. Comparison of the gene expression profiles in THLE-3 cells exposed to these five mutagens at one dose and one time point. Venn diagram shows the gene expression profiles; AF-2, MNU, MMS, 4-NQO and 2NF up regulated 1,026, 1,120, 500, 560 and 446 genes and also down regulated 1,041, 872, 325, 515 and 408 genes, respectively (Figure 2).

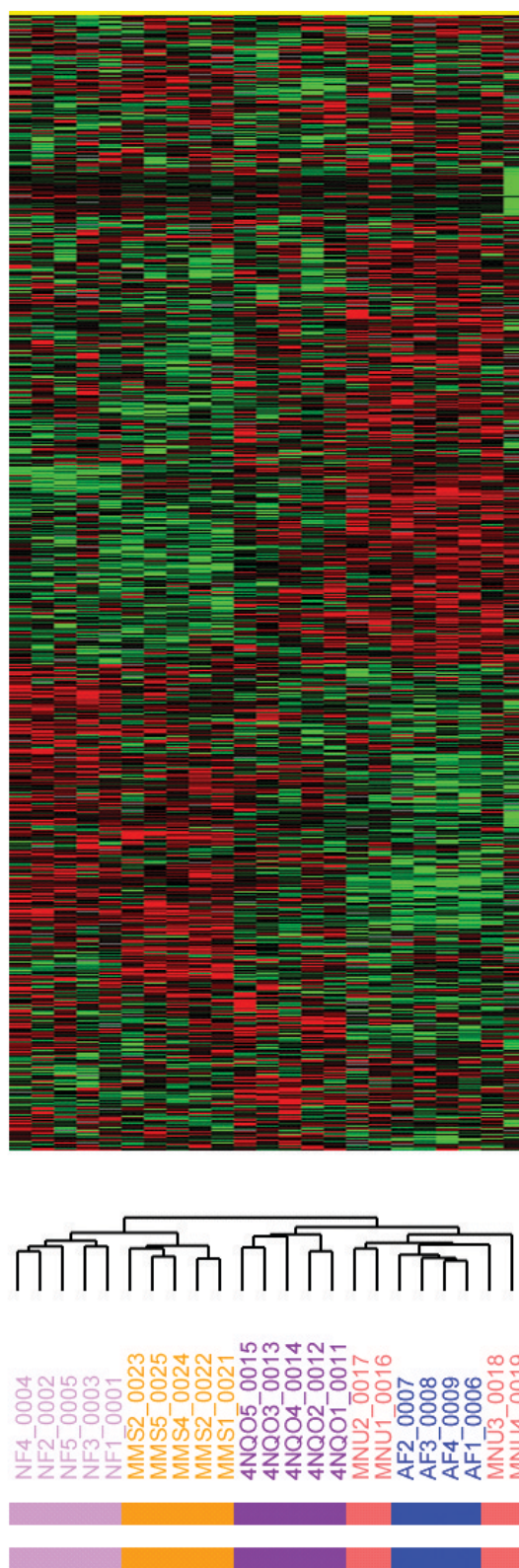There were 51 up-regulated common genes and 45 down-regulated common genes through AF-2, MNU,

Figure 1. Hierarchical cluster image showing the differential gene expression profiles of 5 mutagens exposed in THLE-3 cells.

(a)

1.5 fold over expression
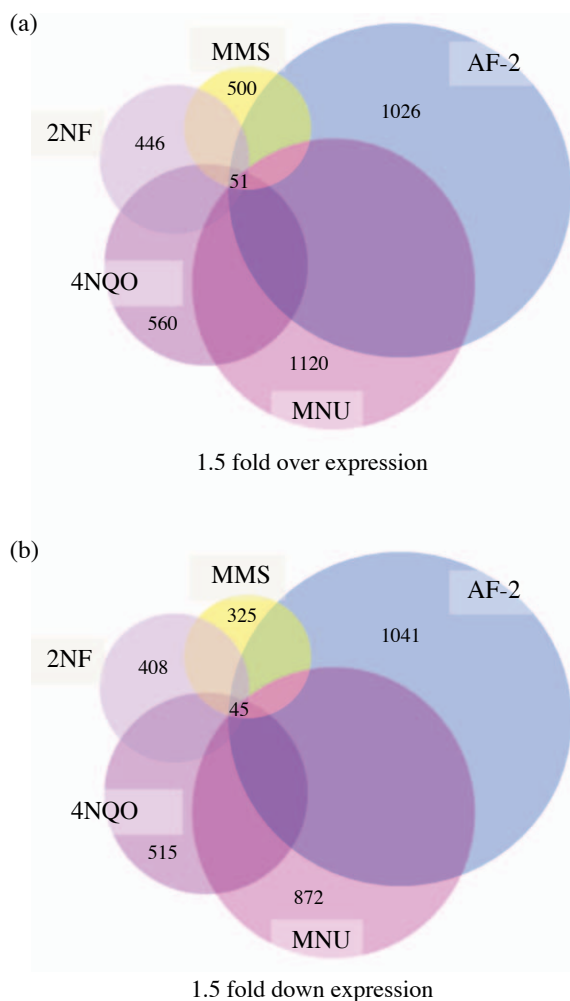
(b)

1.5 fold down expression

Figure 2. Venn diagram showed the differentially expressed genes by 5 mutagens. The diagrams were generated from the list of up (a) and down (b) regulated genes that were >1.5 fold with microarray analysis.

MMS, 4-NQO and 2NF exposure. Table 1 showed the list of the up-regulated common genes and Table 2 showed the down-regulated common genes, which statistically significantly changed their expression profiles, along with their major functions like cell cycle, apoptosis, signal transduction and transport.

Discussion

As state previously, mutagen is a agent that increases the frequency of mutations occurred due to DNA damage and exposure to DNA damaging agents can elicit a variety of stress-related responses that may alter the expression of genes associated with numerous biological pathway leading to the enhance-

ment of DNA repair, growth arrest to delay cell cycle (enabling repair of damage) or induction of apoptosis[12]. We can confirm this process by gene expressions with mutagens exposure. Microarray technique is one of the promising tools for the identification of expression profiles. In this study, we have used this approach to identify the gene expression profiles induced by 5 mutagens in THLE-3 cells using 19 k whole human oligonucleotide microarray. Quintuplicate assays were performed for each chemical to avoid the error rates. From microarray study with statistical analysis, AF-2, MNU, MMS, 4-NQO and 2NF up-regulated 1,026, 1,120, 500, 560 and 446 genes and also down-regulated 1,041, 872, 325, 515 and 408 genes, respectively, and there were 51 up-regulated common genes and 45 down-regulated common genes (Figure 2). Although many types of gene expression changes were noted in our studies, the focus of this discussion will be focused on gene expression changes that are involved in apoptosis, cell cycle, signal transduction and transport to evaluate following process by mutagen effect. The first step in the initiation of activity of DNA damage by mutagens is recognition of DNA damage by sensor genes. Next, signals are transmitted to transducers by mediator genes. And the regulated transducer genes suppress effecter genes, finally arresting the cell cycle and inducing apoptosis[13]. These pathways include so many factors involved in DNA damage and following steps. We can refer these factors as signal transduction genes because recognizing, mediating and transmitting are all part of signal transduction. Our gene expression data have several genes involved major functions like apoptosis (TP73L, ALOX15B), cell cycle (E2F5, MST016, IFITM1), signal transduction (EMR1, CYTL1, CX3CR1, RHOH, VAV2, CD79A, DCDC2, TNFSF8), transport (SLC25A5, APOD, KCNQ3, ABCC6, LOC644353, TCOF1, HPX, ATP2B2, SLC9A1) (shown in Table 1 and 2). From that remark, we can infer that 5 mutagens cause DNA damage and following apoptosis and cell cycle arrest.

In conclusion, although this data is not enough to say the mechanistic inside of genotoxicity of mutagens, we have identified differentially expressed genes in THLE-3 cells treated to AF-2, MNU, MMS, 4-NQO and 2NF. These genes could be a promising biomarker to detect other mutagens induced DNA damage. Also, it can be suggested that oligonucleotide microarray is an efficient technology for evaluating the gene regulation by mutagens exposure and this approach also offers the possibility to identify the molecular markers.

Table 1. List of up-regulated common genes by five mutagens in THLE-3 cells.

Accession no.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)				
			4NQO	AF-2	MMS	MNU	2NF
Apoptosis							
NM_003722	TP73L	tumor protein p73-like	2.65	2.49	2.09	2.44	2.35
Cell cycle							
NM_001951	E2F5	E2F transcription factor 5, p130-binding	2.44	3.80	2.19	3.81	2.20
AF110322	MST016	MSTP016	1.54	1.79	1.66	2.56	1.55
Transcription regulation							
NM_006940	SOX5	SRY (sex determining region Y)-box 5	1.54	2.26	1.59	2.66	1.65
NM_005461	MAFB	v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian)	4.22	5.95	1.56	7.56	1.68
AF211977	LENG10	Leukocyte receptor cluster (LRC) member 10	2.58	2.26	3.40	2.54	2.24
NM_002309	LIF	leukemia inhibitory factor (cholinergic differentiation factor)	1.71	1.67	4.13	2.44	1.85
AK027024	SII	Transcription elongation factor A , 3	2.50	3.54	2.76	3.94	1.93
AL049434		putative homeodomain transcription factor 1	2.50	2.85	3.25	1.62	1.74
Immune response							
M26147	DNTT	deoxynucleotidyltransferase	1.95	3.25	1.84	5.25	2.20
NM_001643	APOA2	Homo sapiens apolipoprotein A-II, mRNA.	1.89	2.38	1.05	2.20	1.57
Signal transduction							
NM_001974	EMR1	egf-like module containing, mucin-like, hormone receptor-like 1	1.67	1.94	1.70	2.51	1.52
NM_018659	CYTL1	cytokine-like 1	3.02	3.78	2.62	4.32	2.63
NM_001337	CX3CR1	Homo sapiens chemokine (C-X3-C motif) receptor 1, mRNA.	1.16	2.24	1.18	2.42	1.18
NM_004310	RHOH	Homo sapiens ras homolog gene family, member H, mRNA.	0.86	1.74	0.75	1.59	1.07
Transport							
L32786	SLC25A5	Solute carrier family 25, member 5	2.26	2.49	2.49	1.70	2.89
NM_001647	APOD	apolipoprotein D	1.71	2.24	1.61	2.69	1.81
NM_004519	KCNQ3	potassium voltage-gated channel, KQT-like subfamily, member 3	2.01	1.84	1.84	1.82	2.17
NM_001171	ABCC6	ATP-binding cassette, sub-family C (CFTR/MRP), member 6	2.00	1.98	1.69	2.24	2.11
AF086548	LOC644353	Hypothetical LOC644353	1.52	1.86	1.58	3.33	2.52
AF085917		potassium channel tetramerisation domain containing 1	1.73	2.10	2.31	2.05	1.98
Etc							
AF007132	ABHD5	abhydrolase domain containing 5	1.69	1.57	1.75	1.82	1.89
NM_001936	DPP6	dipeptidyl-peptidase 6	2.60	2.29	1.86	3.01	2.19
BE887356	MRPL9	mitochondrial ribosomal protein l9	1.99	1.86	1.66	1.74	1.79
AK021863	RBM17	RNA binding motif protein 17	1.98	2.67	1.61	1.66	1.86
AK024433	MRPS25	Mitochondrial ribosomal protein S25	2.03	1.77	2.15	2.73	1.82
NM_012190	ALDH1L1	aldehyde dehydrogenase 1 family, member L1	1.70	2.27	1.59	2.18	1.89

Methods

Chemicals and Reagents

Furylfuramide (AF-2), N-nitroso-N-methylurea (MNU), methylmethanesulfonate (MMS), 4-nitroquinoline-N-oxide (4-NQO), 2-nitrofluorene (2NF) and dimethylsulphoxide (DMSO) were obtained from Sigma-Aldrich (USA). BEGM was purchased from Clonetics (USA), Dulbecco's phosphate buffered saline (PBS) and fetal bovine serum (FBS) were the products of GIBCO™ (USA). All other chemicals used were of analytical grade or the highest grade available.

Cell Lines and Culture

Human normal hepatocytic cell line (THLE-3) was used throughout the study, purchased from ATCC (USA). The cells were grown in BEGM medium supplemented with 10% inactivated FBS, 70 ng/mL phosphoethanolamine at 37°C in 5% CO_2 atmosphere. For cell growth, the medium was renewed every two

Table 2. List of down-regulated common genes by 5 mutagens in THLE-3 cells.

Accession no.	Gene symbol	Gene name	Mean intensity (Cy5/Cy3)				
			4NQO	AF-2	MMS	MNU	2NF
Apoptosis							
NM_001141	ALOX15B	arachidonate 15-lipoxygenase, type B	0.44	0.35	0.54	0.33	0.50
Cell cycle							
NM_003641	IFITM1	interferon induced transmembrane protein 1 (9-27)	0.43	0.34	0.45	0.47	0.57
Transcription regulation							
S72620	FLI1	Friend leukemia virus integration 1	0.51	0.47	0.52	0.38	0.64
NM_006399	BATF	basic leucine zipper transcription factor, ATF-like	0.60	0.58	0.64	0.65	0.58
M26747	THRB	Thyroid hormone receptor, beta (erythroblastic leukemia viral (v-erb-a) oncogene homolog 2, avian)	0.60	0.44	0.40	0.54	0.35
X99631	HOXC12	H.sapiens mRNA for HOXC12 protein, exon 2	0.49	0.29	0.64	0.41	0.66
Z36807	MAML3	mastermind-like 3 (Drosophila)	0.54	0.49	0.54	0.48	0.50
X52339	KOX8	Zinc finger protein 708	0.37	0.22	0.54	0.14	0.64
Immune response							
NM_000715	C4BPA	complement component 4-binding protein, alpha)	0.20	0.43	0.35	0.49	0.28
Signal transduction							
NM_003371	VAV2	vav 2 oncogene	0.57	0.43	0.50	0.40	0.46
NM_001783	CD79A	CD79A antigen (immunoglobulin-associated alpha)	0.49	0.31	0.40	0.38	0.48
NM_003394	WNT10B	wingless-type MMTV integration site family, member 10B	0.37	0.40	0.51	0.22	0.38
NM_016356	DCDC2	Homo sapiens doublecortin domain containing 2, . mRNA	0.55	0.53	0.47	0.48	0.56
NM_001244	TNFSF8	Homo sapiens tumor necrosis factor (ligand) superfamily, member 8 mRNA	0.39	0.22	0.48	0.19	0.44
Transport							
NM_000356	TCOF1	Treacher Collins-Franceschetti syndrome 1	0.55	0.55	0.62	0.54	0.66
J03048	HPX	hemopexin mRNA	0.29	0.17	0.48	0.33	0.42
NM_001683	ATP2B2	ATPase, Ca++ transporting, plasma membrane 2	0.26	0.14	0.59	0.24	0.37
M81768	SLC9A1	Solute carrier family 9 (sodium/hydrogen exchanger), member 1	0.55	0.37	0.64	0.46	0.53
Etc							
AF000562	UPK2	uroplakin II mRNA	0.64	0.58	0.59	0.63	0.48
NM_015900	PLA1A	phospholipase A1 member A	0.61	0.46	0.64	0.27	0.64
NM_014509	SERHL2	Serine hydrolase-like 2	0.45	0.37	0.57	0.33	0.46
AF118072	ENSG00000184150	Homo sapiens PRO1716 mRNA	0.61	0.60	0.44	0.53	0.62
AB014541	AATK	Apoptosis-associated tyrosine kinase	0.66	0.38	0.62	0.46	0.60
AJ008151	MAP3K14	Mitogen-activated protein kinase kinase kinase 14	0.46	0.37	0.47	0.36	0.62
NM_015950	MRPL2	Mitochondrial ribosomal protein L2	0.56	0.47	0.62	0.48	0.43
S49432	COL6A3	type VI collagen alpha 3 chain	0.41	0.43	0.55	0.43	0.29

or three days. Approximately 80% confluence achieved by plating 1×10^6 cells/mL in 100 mm culture dish (Falcon, USA).

Determination of Cell Viability

MTT assay was performed for the detection of cell viability[14]. A 24-well palte was used for cytotoxicity assay. Cells were seeded at a seeding density of 3×10^4 cells/mL on a well in 500 μL of media. Cells were exposed to various concentrations of AF-2, MNU, MMS, 4-NQO and 2NF in culture medium at 37°C for 3 hrs exposure time. Seventy-five μL of MTT (5 mg/mL in PBS) solution was added to each well and incubated for 3 hrs. DMSO solution was added to each

tube and transfer in 96 well plate. The optimal density (OD) of the purple formazan product was measured at a wavelength of 540 nm. The IC_{20} of cell proliferation in a particular chemical was defined as the concentration that causes a 20% reduction in the cell viability versus the solvent treated control. The IC_{20} values were directly determined from the linear dose-response curves.

RNA Extraction

Total RNA was extracted from the THLE-3 cells treated to 243.12 μM, 2,093.97 μM, 510.37 μM, 0.83 μM and 80.78 μM for AF-2, MNU, MMS, 4-NQO and 2NF, respectively, for 3 hrs using the Trizol rea-

gent (Invitrogen, USA) and purified using RNeasy mini kit (Qiagen, USA) according to the manufacturer's instructions. Genomic DNA was removed using RNase-free DNase set (Qiagen, USA) during RNA purification. The amount of each total RNA concentration was quantified using SmartSpec 3000 (Bio-Rad, CA). Only samples with an A260/A280 ratio between 1.9 and 2.2 were considered for suitable use and its quality was checked by Bioanalyzer 2001 (Agilent Technologies Inc, USA).

Oligonucleotide Microarray Hybridization

Gene expression analysis was conducted on the RNA samples using 19 k whole human genome microarray (Catholic University, Korea). Quintuplicate analysis was performed for each chemical simultaneously. In brief, RNA was primed with 1 μg of oligo (dT) primer (Invitrogen) at 65°C for 10 min and reverse transcription was followed by adding 21 μL of master mix solution: 8 μL of 5X first strand buffer, 4 μL of 0.1 M DTT, 2 μL of 20X dNTP (10 mM dATP, dCTP, dGTP and 4 mM dTTP), 4 μL of 1 mM Cy3-dUTP (MEN) or Cy5-dUTP (MEN) and 2 μL of Superscript™ II (200 U/μL, Invitrogen). The Cy5-and Cy3-labeled targets were then combined and purified with a Microcon® YM-30 Column (Milipore). The volume of purified targets were adjusted to 16 μL, and then mixed with 40 μg of Herring Sperm DNA and 20 μL DIG Easy hybridization solution (Roche). The targets were then hybridized onto spotted-oligoarrays at 42°C for overnight. Washed arrays were scanned by using GenePix 4000B (Axon Instruments, USA) and Cy3/Cy5-signals were measured by using a GenePix Pro 4.1 (Axon Instruments, USA).

Data Analysis

The fluorescent intensity of each spot was calculated by local median background subtraction. The robust scatter-plot smoother LOWESS function was used to perform intensity dependent normalization for the gene expression. Scatter plot analysis was made by Microsoft Excel 2000 (Microsoft, USA). Significance Analysis of Microarray (SAM) was performed for the selection of the genes with significant gene expression changes[15]. Computing a *q*-value for each gene assessed the statistical significance of the differential expression of genes. To determine the *q*-value, a permutation procedure was used and for each permutation, two-sample *t* statistics were computed for each gene. Genes were considered differentially expressed when logarithmic gene expression ratios in three independent hybridizations were more than 1.5 or less than 0.50, i.e., 1.5-fold difference in expression level, and when the *q*-values were < 5.

Functional Grouping and Clustering Analysis

In order to classify the selected genes into groups with a similar pattern of expression, each gene was assigned to an appropriate category according to its main cellular function. The categories included apoptosis, cell cycle, transcription regulation, immune response, signal transduction and transport. The necessary information to categorize each gene was obtained from several databases particularly the database located at http://www.david.abcc.ncifcrf.gov/tools.jsp/database.

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