MOLECULAR & CELLULAR TOXICOLOGY, Vol. 3, No. 3, 165-171, September 2007

Toxicogenomics Study on TK6 Human Lymphoblast Cells Treated with Mitomycin C

Joo Hwan Kim[1], Ye Mo Koo[1], Woo Sun Lee[1], Soo Kyung Suh[1], Jin Seok Kang[1], Euisik Han[1], Seung Hee Kim[1] & Sue N. Park[1]

[1]Department of Toxicological Researches, National Institute of Toxicological Research, Korea Food and Drug Administration, 194 Tongil-ro, Eunpyeong-Gu, Seoul 122-704, Korea
Correspondence and requests for materials should be addressed to S. N. Park (suenie@kfda.go.kr)

Accepted 10 August 2007

Abstract

Mitomycin C (MMC), an antitumor antibiotic isolated from *Streptomyces caespitosus*, is used in chemotherapy of gastric, bladder and colorectal cancer. MMC is activated *in vivo* to alkylate and crosslink DNA, via G-G interstrand bonds, thereby inhibiting DNA synthesis and transcription. This study investigates gene expression changes in response to MMC treatment in order to elucidate the mechanisms of MMC-induced toxicity. MMC was admistered with single dose (0.32 and 1.6 μM) to TK6 cells. Applied Biosystem's DNA chips were used for identifying the gene expression profile by MMC-induced toxici-ty. We identified up- or down-regulated 90 genes including cyclin M2, cyclin-dependent kinase inhibitor 1A (p21, cip1), programmed cell death 1, tumor necrosis factor (ligand) superfamily, member 9, *et al*. The regulated genes by MMC associated with the biological pathways apoptosis signaling pathway. Further characterization of these candidate markers related to the toxicity will be useful to understand the detailed mechanism of action of MMC.

Keywords: Toxicogenomics, Mitomycin C, Microarray, Gene expression

The screening of chemical compounds for hazardous properties relies on a few *in vitro* assays and on acute to chronic studies with animal models. Despite their frequent use, the reliability, relevance and effectiveness of these methodologies are continuously questioned[1]. Toxicogenomics provides novel mechanistic insight into the response of cells to DNA damage by developing a more complete picture of toxicologically important events as opposed to the historical method of examining the response of a few, select genes[2]. Toxicant-specific gene expression signatures will provide biomarkers for predicting the toxicity of toxic chemicals, detect toxicity at levels that do not yield clinical symptoms, and provide insights into mechanisms of toxicity[3].

Mitomycin C (MMC), and antitumor antibiotic isolated from *Streptomyces caespitosus*, is used in chemotherapy of gastric, bladder and colorectal cancer. MMC is activated *in vivo* to alkylate and crosslink DNA, via G-G interstrand bonds, thereby inhibiting DNA synthesis and transcription[4].

Several enzymes, including cytosolic (NADPH: quinine oxidoreductase 1 (NQO1) and other unknown cytosolic proteins) and microsomal (P-450 reductase, cytochrome b5 reductase, xanthine oxidoreductase and dehydrogenase), have been shown to catalyze MMC activation, leading to DNA cross-linking and cytotoxicity[5]. Treatment of mammalian cells with MMC caused an increase in the cellular p53 level[6]. In cells, MMC is enzymatically reduced, yielding reactive species that are capable of producing radicals through redox cycling, as well as a variety of DNA adduct. Six major adducts are formed as shown in mouse mammary tumor cells and their precise molecular structures have been elucidated[7].

In this study, we evaluated the gene expression changes and DNA damage in human lymphoblast TK6 cells exposed to MMC using microarray. Human lymphoblast TK6 cells were selected for these studies because they are a commonly used cell line for genetoxicity assessments because of TK6 cells are heterozygous for the thymidine kinase (*tk*) gene and wild-type for the *p53* gene.

Cytotoxicity Test

Cytotoxicity of test chemicals in TK6 cells was examined to determine optimal concentration. The survival percentage relative to control was determined as a percentage of the number of cells survived after treatment. Cell viability was tested after 4 h with each concentration of test chemicals using the MTS assay (Figure 1). The 50% inhibitory concentration (IC_{50}) of MMC was 3.1 μM. The highest concentration was selected 20-30% cytotoxiciy and low concentration was selected 3-10% cytotoxicity. The selection of concentration allowed to evaluate the effect of cyto-

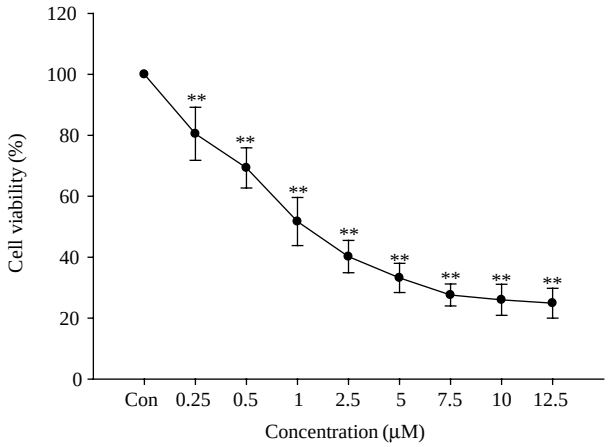

Figure 1. The effects of *in vitro* treat to mitomycin C on cell viability of TK6 cells assayed by MTS. Each value represents mean ± S.D. **: Different significant from control ($P < 0.001$)

toxicity on the gene expression profiles.

Microarray Test and Gene Expression Change

ABI Genome Microarray chip (human) (Applied Biosystems, USA) were used to measure stress-associated gene expression changes in human lymphoblast TK6 cells. We wanted to examine signs of stress -associated response. The RNA was isolated after 4 h exposure with MMC. The gene expression profiles were measured in cells treated with concentration 0.32 and 1.6 μM.

The analysis of gene expression date identified 90 genes that exhibited statistically significant gene expression changes (Table 1-4). Among the altered genes, there were cyclin M2, cylin-dependent kinase inhibitor 1A (p21, cip1), programmed cell death 1, tumor necrosis factor (ligand) superfamily, member 9, *et al*. Hierarchical clustering analysis was performed with 90 differentially expressed genes according to their similarity in expression pattern (Figure 2).

Gene ontology biological process, molecular function and pathway mapping were performed using Panther Database (http://www.pantherdb.org) to evaluate the expression pattern on basis of gene function and pathway. The result showed significantly changed genes were related to apoptosis signaling pathway (Figure 3, Table 5)

Table 1. Representative list of differentially expressed genes by mitomycin C in TK6 cells (Continued).

Gene name (Up-regulated genes)	Fold change	
	0.32 μM	1.6 μM
Activationg transcription factor 3	1.52	3.20
AFG3 ATPase family gene 3-like 1 (yeast)	3.99	3.72
carbohydrate (chondroitin 4) sulfotransferase 13	1.66	1.89
carbohydrate (N-acetylglucosamine 6-O) sulfotransferase 6	1.82	1.59
cholinergic receptor, muscarinic 4	3.41	1.65
chromosome 21 open reading frame 22	2.01	2.16
coiled-coil domain containing 24	1.58	1.69
cyclin-dependent kinase inhibitor 1A (p21, Cip1)	1.65	5.30
deiodinase, iodothyronine, type III	1.97	2.12
deltex homolog 2 (Drosophila)	2.12	1.60
endothelin 2	2.18	2.51
family with sequence similarity 91, member A2	3.59	1.88
feline sarcoma oncogene	3.09	1.73
G protein-coupled receptor 103	1.58	1.51
G protein-coupled receptor 172B	1.85	2.71
gap junction protein, beta 4 (connexin 30.3)	2.20	2.41
gap junction protein, chi 1, 31.9 kDa (connexin 31.9)	2.27	2.16
golgi autoantigen, golgin subfamily a, 8B|golgi autoantigen, golgin subfamily a, 8A	1.65	1.85
golgi autoantigen, golgin subfamily a, 8B|golgi autoantigen, golgin subfamily a, 8A	1.99	1.85
heparan sulfate (glucosamine) 3-O-sulfotransferase 4	2.03	1.87
hexokinase 3 (white cell)	3.00	2.47
hydrocephalus inducing	2.42	1.71
keratin 15	2.04	1.81
keratin associated protein 4-15	1.98	1.88
LBXCOR1 homolog (mouse)	2.78	2.35
MADS box transcription enhancer factor 2, polypeptide B (myocyte enhancer factor 2B)	1.72	1.51
Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	1.77	4.01
neurogenic differentiation 2	3.35	2.11

Discussion

Genotoxic stress causes a variety of cellular responses including the transcriptional activation of genes regulating DNA repair, cell cycle arrest, and apoptosis[8]. Although all genotoxins produce such general responses, the mechanisms governing response to divergent forms of DNA damage are potentially diverse themselves. The cellular responses to

Table 2. Representative list of differentially expressed genes by mitomycin C in TK6 cells (Continued).

Gene name (Up-regulated genes)	Fold change	
	0.32 μM	1.6 μM
olfactory receptor, family 13, subfamily J, member 1	2.31	1.57
peroxisomal membrane protein 4, 24 kDa	2.02	2.68
programmed cell death 1	2.76	1.99
protein phosphatase 2, regulatory subunit B (B56), beta isoform	1.55	1.82
protein tyrosine phosphatase, receptor type, C	2.94	2.75
reticulon 4 receptor-like 1	1.75	1.60
REX1, RNA exonuclease 1 homolog (S. cerevisiae)	2.21	2.28
Rho GTPase activating protein 30	1.99	2.21
ring finger protein 183	2.04	1.88
RNA pseudouridylate synthase domain containing 1	1.76	1.55
S100 calcium binding protein A7 (psoriasin 1)	3.18	1.51
SH3 and multiple ankyrin repeat domains 3	2.78	2.14
snail homolog 1 (Drosophila)	1.68	1.86
solute carrier family 9 (sodium/hydrogen exchanger), member 3 regulator 2	2.98	3.42
spermine oxidase	1.66	1.53
splicing factor 3a, subunit 3, 60 kDa	6.90	2.88
suppressor of cytokine signaling 3	3.02	2.30
Theg homolog (mouse)	1.70	3.11
tight junction protein 3 (zona occludens 3)	1.71	1.81
tripartite motif-containing 3	2.96	1.69
tubby homolog (mouse)	3.18	2.63
tumor necrosis factor (ligand) superfamily, member 9	1.54	2.12
uroplakin 2	2.67	1.91
variable charge, X-linked 2	1.65	3.22
zinc finger protein 197	1.97	1.62
Zinc finger protein 75 (D8C6)	1.94	1.94

Table 3. Representative list of differentially expressed genes by mitomycin C in TK6 cells (Continued).

Gene name (Up-regulated genes)	Fold change	
	0.32 μM	1.6 μM
Adenylate cyclase 7	−1.80	−1.52
amyloid beta (A4) precursor protein-binding, family A, member 2 (X11-like)	−4.15	−2.26
bol, boule-like (Drosophila)	−2.03	−2.31
bolA-like 1 (E. coli)	−1.94	−1.87
calcium channel, voltage-dependent, beta 2 subunit	−1.88	−2.90
chromosome 8 open reading frame 53	−1.81	−1.82
chromosome 9 open reading frame 116	−1.59	−1.59
cyclin M2	−1.65	−2.71
EPH receptor B1	−2.08	−1.94
family with sequence similarity 100, member B	−1.74	−1.65
GTF2I repeat domain containing 1	−1.59	−1.63
histone 2, H2be	−1.70	−3.79
histone deacetylase 4	−1.66	−1.53
homeobox B4	−1.62	−1.66
interferon-induced protein with tetratricopeptide repeats 1	−2.28	−1.51
lectin, galactoside-binding, soluble, 12 (galectin 12)	−2.87	−3.64
leucine-rich repeat kinase 1	−2.00	−1.87
low density lipoprotein receptor-related protein 6	−1.52	−1.63
microtubule associated serine /threonine kinase 3	−2.06	−1.50
neutrophil cytosolic factor 4, 40 kDa	−2.09	−1.68
RALBP1 associated Eps domain containing 2	−2.06	−1.70
Rap guanine nucleotide exchange factor (GEF) 5	−1.65	−1.79
S100 calcium binding protein A2	−2.14	−1.61
serine dehydratase-like	−2.65	−1.56
Sp8 transcription factor	−2.22	−2.44
T-cell activation GTPase activating protein	−1.56	−1.66
transducin-like enhancer of split 6 (E(sp1) homolog, Drosophila)	−3.94	−1.99
transmembrane and coiled-coil domain family 1	−1.91	−1.66

genotoxic stress are partly mediated by the activation of signal transduction pathways involving mitogen-activated protein kinases (MAPKs), including the extracellular signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), and the p38 kinases[9,10].

This study demonstrates that expression profiling for 90 selected genes in cells following exposure to MMC. We investigated the MMC-induced changes in gene expression profiles using the human lymphoblast TK6 cell line Analyzing gene expression, we identified a total of 90 genes with statistically significant changes in expression after treatment of MMC.

van Delft J. H. M. *et al*. showed that methylating agents (MMC, methyl methanesulfonate (MMS)) are

Table 4. Representative list of differentially expressed genes by mitomycin C in TK6 cells.

Gene name (Up-regulated genes)	Fold change	
	0.32 μM	1.6 μM
tubulin tyrosine ligase	−1.53	−1.64
tubulin tyrosine ligase-like family, member 4	−1.95	−1.78
tumor necrosis factor (ligand) superfamily, member 9	1.54	2.12
zinc and ring finger 1	−1.67	−3.08
zinc finger protein 615	−2.05	−1.69

much smaller and hardly distort the DNA-helix, and thereby trigger less genetic pathyway. In deed, the p53 target genes for DNA damage response BAX, CDKN1A, CADD45 and PCNA are all up-regulated by methylating agents except any methylating agents. Recently, study has been comparison of supervised clustering methods to discriminate genotoxic (GTX) from non-genotoxic (NGTX) carcinogens[11]. Both 24 h after treatment of human HepG2 cells, gene expression profiles could correctly discriminate between both classes of carcinogens by 4 different supervised clustering methods, namely Pearson correlation analysis, nearest shrunken centroids analysis, K-nearest neighbour analysis and Weighted voting. One gene is selected in all models, namely BAX, which in general is induced by GTX carcinogens and suppressed by NGTX carcinogens. BAX is involoved in the induction of apoptosis, and its expression is induced in the DNA damagen response pathway by the tumor suppressor protein p53[12].

The gene expression profile provides us a better understanding of underlying mechanisms for MMC-induced toxicity. Integration of gene expression changes with known pathological changes can be used to formulate a mechanistic scheme for MMC-induced toxicity as Figure 3 and Table 5.

We conclude that there has been interest in using microarray in toxicology to quickly evaluate toxicants based on characteristic expression profiles, but it should be pointed out that additional experiment may be necessary to further confirm the utility of these gene changes as biomarkers of exposures to agents that induce DNA damage through the above mechanism.

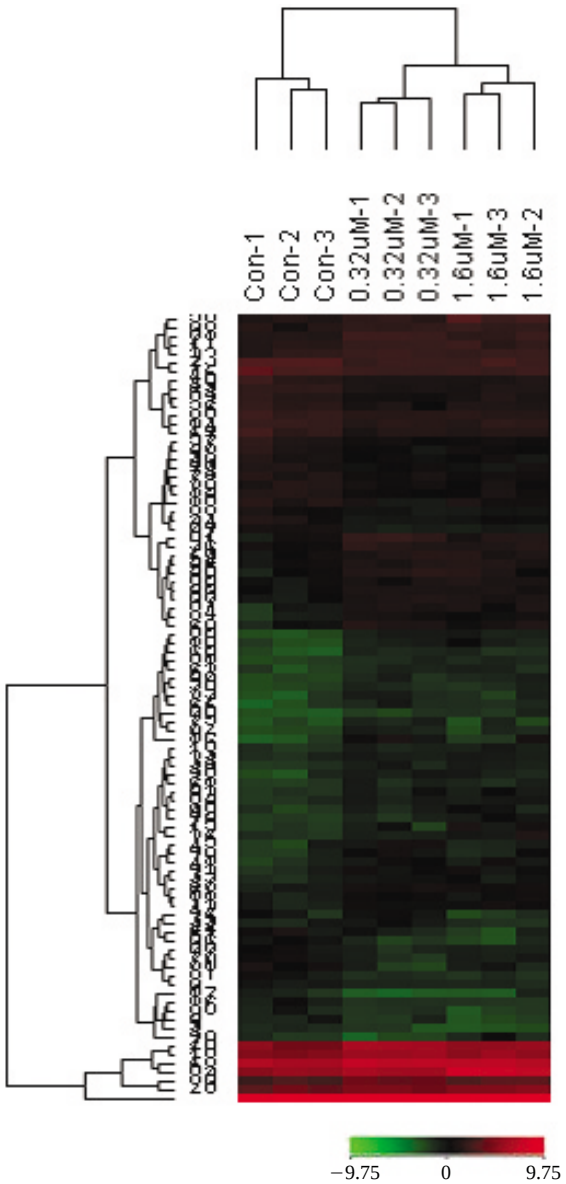

Figure 2. Hierarchical cluster image showing the gene changes occurring in TK6 cells treated mitomycin C after 4 h.

Methods

Materials

Mitomycin C, glucose, sodium pyruvate, sodium carbonate and DMSO were obtained from Sigma (St. Louis, Mo). RPMI medium 1640, FBS, penicillin-streptomycin were obtained from Gibco BRL (USA). MTS (3-4-5-dimethylthiazol-2-yl)-5-(3-carboxy methoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium was purchased from Promega (USA).

Cell Culture

Human lymphoblast TK6 cell line was derived from the parental human lymphoblastoid line HH4 and heterozygous for thymidine kinase (TK), and enzyme which phosphorylates thymidine and its toxic

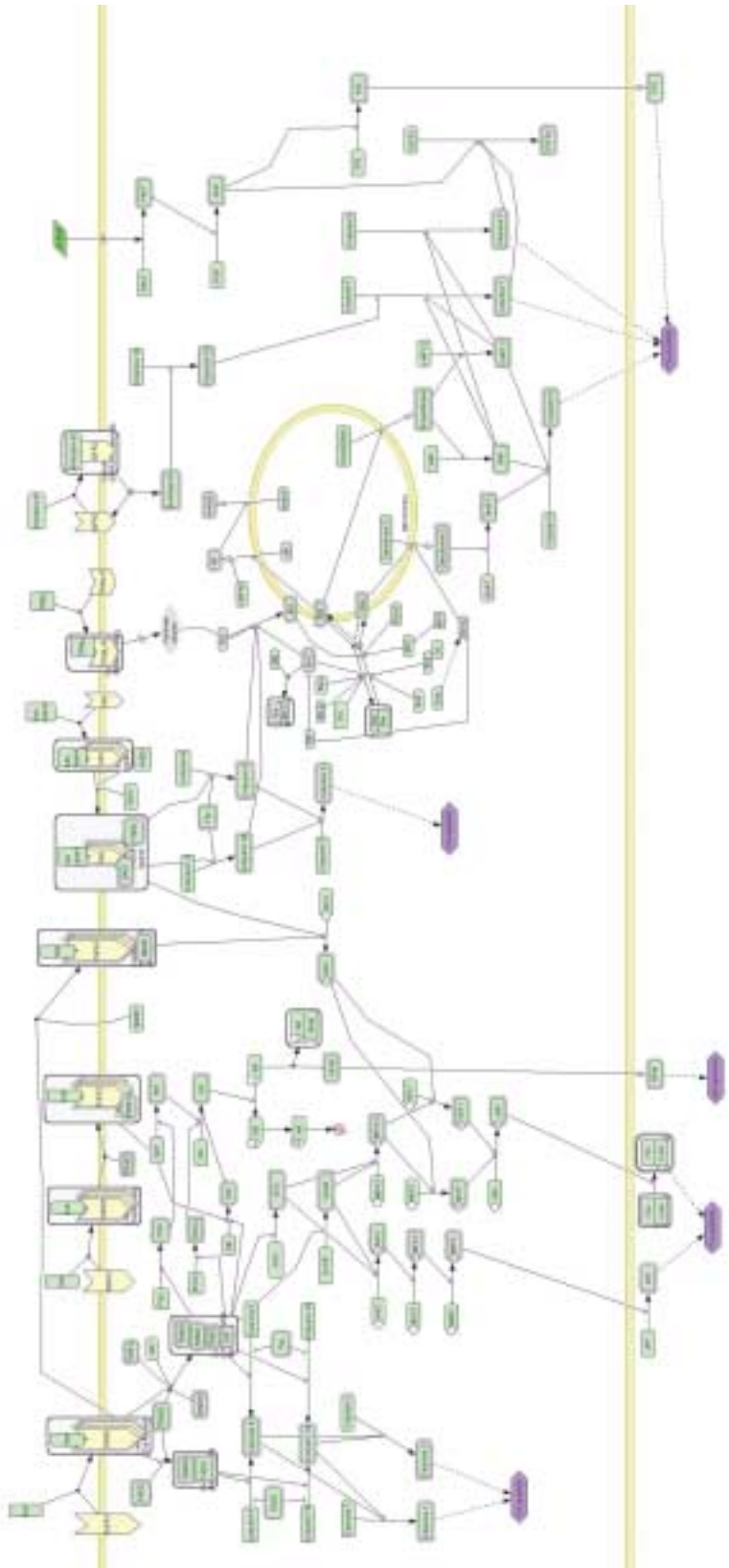

Figure 3. Apoptosis signaling pathway of differentially expressed genes by mitomycin C in TK6 cells.

Table 5. Pathway information of expressed genes by mitomycin C.

Pathway*	NCBI : H. sapiens gene (REF)	Involved gene count	Involved gene/ Total count (%)
Wnt signaling pathway	349	7	2.0%
Apoptosis signaling pathway	131	3	2.3%
Interleukin signaling pathway	194	5	2.6%
P53 pathway	136	2	1.5%

*Panther database system (http://www.pantherdb.org).

analogues in an ATP-dependent reaction. Cells were maintained in RPMI 1640 medium supplemeneted with 10% Fetal Bovine Serum, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose and 1.5 g/L sodium bicarbonate and maintained in 37˚C incubators with a 5% CO_2 atmosphere.

Cytotoxicity

Cytotoxicity was determined by MTS assay. Cells were treated with 0.32 and 1.6 μM concentration of MMC for 4 h. After incubation of the cells for 4 h in MMC containing medium a sample of the culture was mixed with MTS (333 μg/mL) and PMS (25 μM) mixture solution. After 4 h, O.D. was measured at 490 nm.

RNA Preparation

At 4 h post exposure, aliquots of cells were pelleted by centrifugation and washed with phosphate buffered saline. The cell pellets 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, US) and confirmed that 28s/18s ration was between 1.8 and 2.0 and 260/280 nm ratio between 2.0 and 2.2, respectively.

Microarray Analysis

RNA was hybridized to the Applied Biosystems Human Genome Survey Microarray 2.0. Digoxigenin -UTP Labeled cRNA was generated and linearly amplified from 1 μg of pooled-total RNA from each group using Applied Biosystems Chemiluminescent RT-IVT Labeling Kit according to the manufacturer's protocol. Array Hybridization, chemiluminescence detection, image acquisition and analysis were performed using Applied Biosystems Chemiluminescene Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer.

Analysis of Differentially Expressed Genes

Microarray data were analyzed using the software Avadis 3.3 prophetic (Strand Genomics Pvt Ltd.). The local background was subtracted from the raw expression values for all spots. The ratios were then log -transformed ratio equaled to zero. The gene expression ratios were median-centered across all samples. The expression ratio of each gene was made by dividing the normalized values of a gene in chemical treatment group by gene expression mean value in vehicle control group at 4 h. The genes indicating more than 1.5-fold change were usually taken into consideration for data analysis. We used the supervised analysis method for differentially expressed genes between control and treatment group at 4 h. Analysis of gene expression was performed using the Avadis software program. The permutation-based modified t-test was used to provide further confidence in these results. Differential gene expression was analyzed using a two-sample Welch Banjamin Bochberg t-statistic. Thus, differential gene expression associated with each group was tested using significance analysis of microarray. For analysis of data correlation, correspondence analysis (COA) was also performed using the AB1700 package. Hierarchical cluster analysis partitions the data into discrete hierarchical groups based on the trends of the data. The resulting gene lists were basically limited to the genes with the ratio changing values of fold over 1.5 and $P < 0.05$.

Acknowledgements

This work was supported by a research grant, 06131 _OMICS_402 (2006) to Sue Nie Park from the Korea Food and Drug Administration.

References

1. Tennant, R. A. The National Center for toxicogenomics: using new technologies to inform mechanistic toxicology. *Environ Health Perspect* **107**:A256-A258 (2002).
2. Aardema, M. J. & MacGregor, J. T. Toxicology and genetic toxicology in the new era of "toxicogenomics"; impact of "-omics" technologies. *Mutat Res* **499**:13-25 (2002).
3. Newton, R. K., Aaraderma, M. & Aubrecht, J. The utility of DNA microarrays for characterizing genotoxicity. *Environ Health Perspect* **112**:420-422 (2004).
4. Cheung, R. Y., Rauth, A. M. & Wu Yu. In vivo efficacy and toxicity of intratumorally delivered mitomycin C and its combination with doxorubicin using microsphere formulations. *Anticancer Drugs* **16**:423-

433 (2005).
5. Joseph, P., Xu, Y. & Jaiswal, A. K. Non-enzymatic and enzymatic activation of mitomycin C: identification of a unique cytosolic activitiy. *Int J Cancer* **65**: 263-271 (1996).
6. Hess, R. *et al*. Nuclear accumulation of p53 in response to treatment with DNA-damaging agents. *Toxicol Lett* **72**:43-52 (1994).
7. Palom, Y. *et al*. Structure of adduct X, the last unknown of the six major DNA adducts of mitomycin C formed in EMT6 mouse mammary tumor cells. *Chem Res Toxicol* **13**:479-488 (2000).
8. Kastan, M. B. *et al*. Participationg of p53 protein in the cellular response to DNA damage. *Cancer Res* **51**:6304-6311 (1991).
9. Wilhelm, D., Bender, K., Knebel, A. & Angel, P. The level of intracellular glutathione is a key regulator for the induction of stress-activated signal transduction pathways including Jun N-terminal protein kinases and p38 kinase by alkylating agents. *Mol Cell Biol* **17**:4792-4800 (1997).
10. Zhu, K. Q. & Zhang, S. J. Involvement of ATM/ATR -p38 MAPK cascade in MNNG induced G1-S arrest. *World J Gastroenterol* **9**:2073-2077 (2003).
11. van Delft, J. M. H. *et al*. Discrimination of genotoxic from non-genotoxic carcinogen by gene expression profiling. *Carcinogenesis* **25**:1265-1276 (2004).
12. Levine, A. J. p53, the cellular gatekeeper for growth and division. *Cell* **88**:323-331 (1997).