MOLECULAR & CELLULAR TOXICOLOGY, Vol. 3, No. 2, 98-106, June 2007

Gene Expression Profiling of Genotoxicity Induced by MNNG in TK6 Cell

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Accepted 1 May 2007

Abstract

Genotoxic stress triggers a variety of biological responses including the transcriptional activation of genes regulating DNA repair, cell survival and cell death. In this study, we investigated to examine gene expression profiles and genotoxic response in TK6 cells treated with DNA damaging agents MNNG (N-methyl-N′-nitrosoguanidine) and hydrogen peroxide (H_2O_2). We extracted total RNA in three independent experiments and hybridized cRNA probes with oligo DNA chip (Applied Biosystems Human Genome Survey Microarray). We analyzed raw signal data with R program and AVADIS software and identified a number of deregulated genes with more than 1.5 log-scale fold change and statistical significancy. We indentified 14 genes including G protein alpha 12 showing deregulation by MNNG. The deregulated genes by MNNG represent the biological pathway regarding MAP kinase signaling pathway. Hydrogen peroxide altered 188 genes including sulfiredoxins. These results show that MNNG and H_2O_2 have both uniquely regulated genes that provide the potential to serve as biomarkers of exposure to DNA damaging agents.

Keywords: Toxicogenomics, MNNG, Microarray, Genotoxicity

Genotoxic insults triggers a variety of biological responses including the transcriptional activation of genes regulating DNA repair, cell survival and cell death. Genotoxic compounds cause DNA damage by a variety of mechanisms. Direct-acting chemicals that bind covalently to DNA include DNA alkylating agents, cross-linking agents, and oxygen radicals, such as MMS, MMC, and H_2O_2, respectively[1,2]. Indirectacting genotoxicants, on the other hand, alter the function of cellular proteins, leading to accumulation of endogenous DNA damage. Mammalian cells have a repair system to protect them from DNA damage, including base excision repair, nucleotide excision repair, and recombination repair[3].

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[4]. 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[5].

Our laboratory evaluated the gene expression changes and DNA damage in human lymphoblast TK6 cells exposed to different genotoxins. Human lymphoblast cells were selected for these studies because they are a commonly used cell line for genotoxicity assessments. Cells were exposed to the genotoxins following standard test procedures. Chemicals were selected based on their established mechanism of action. MNNG, an alkylating agent, and hydrogen peroxide, a free radical induced DNA damaging agent were selected as test chemicals. Concentrations tested ranged from a no effect level for cytotoxicity and genotoxicity (as measured by DNA damage) up to clearly genotoxic concentrations that induced around 10-50% cytotoxicity, as in the standard method.

Cytotoxic and Genotoxic Response to MNNG and H_2O_2

Cytotoxicity of test chemicals in TK6 cells was examined to determine the non-toxic concentration on induction of DNA damage. Cell viability was tested after 2 h with concentrations between 0.006 mM and 0.75 mM of MNNG or between 0.01 mM and 5 mM of hydrogen peroxide Using MTS assay (Figure 1).

The IC_{50} of MNNG and hydrogen peroxide were 10 μM, and 100 μM, respectively, in TK6 cells. To avoid complications of gene expression changes due to

differences in cytotoxicity, concentrations were selected for each chemical to insure similar levels of cytotoxicity at each dose. The highest concentration for each chemical was selected on the basis of a comet assay and ~50% cytotoxicity, the mid concentration was ~30% cytotoxicity, and the low concentrationwas 10-20% cytotoxicity. The selection of 50% cytotoxicity allowed comparison of MNNG and H_2O_2 at equitoxic concentrations to evaluate the effect of cytotoxicity on the gene expression profiles. Induction of DNA damage by genotoxicants was evaluated with comet assays. Evidence of significant DNA damage was evident in TK6 cells at all concentrations (Figure 2).

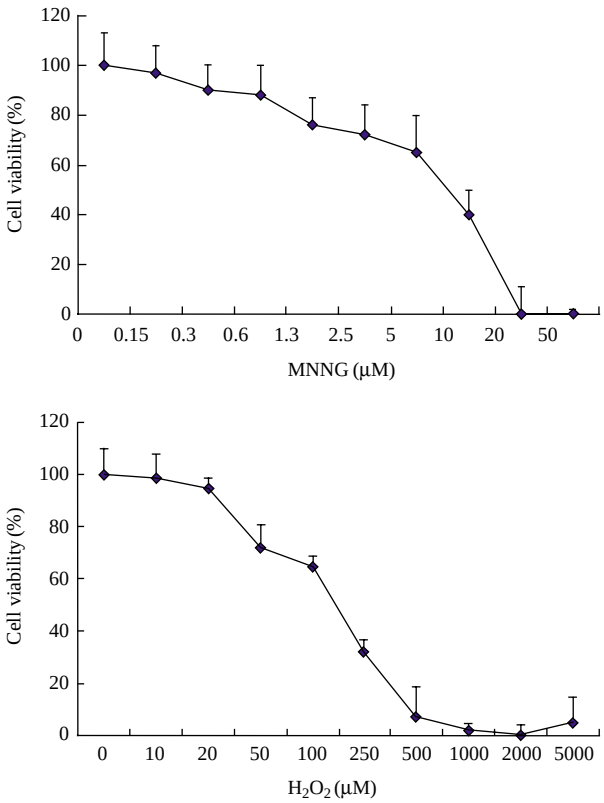

Figure 1. Cell viability tests of TK6 cells after treatment of MNNG and hydrogen peroxide by MTS assay.

Gene Expression Changes

To analyze gene expression profiles of MNNG, we compared toxicants-treated group versus vehicle control group at each concentrations using two way ANOVA methods and identified 447 genes out of total 32,381 probe sets. By statistical criteria of >1.5 fold changes at $P<0.05$ (False discovery rate <0.25), a total of 14 genes were significantly changed in at least one of treatment conditions (Figure 3).

Hierarchical clustering analysis was performed with 14 differentially expressed genes according to their similarity in expression pattern (Figure 4). An abbreviated list of differentially expressed genes according to molecular function and/or biological pathway is presented in Table 1. Among the altered genes, there were guanine nucleotide binding protein, gamma12, 2-aminoadipic 6-semialdehyde dehydrogenase, and aldehyde dehydrogenase 7 family, member A1.

Gene ontology biological process, molecular func-

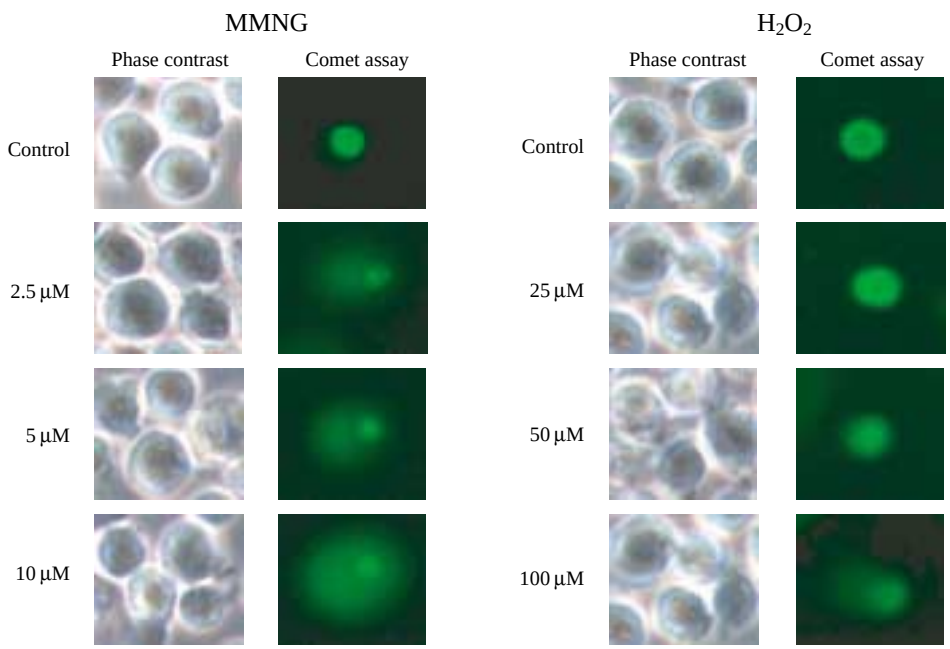

Figure 2. Cell morphology and DNA damage analysis in TK6 cells treated by MNNG. The single cell gel electrophoresis (SCGE) assay, also known as the comet assay, was performed to measure the amount of DNA breakage in the cells.

tion and pathway mapping were performed using KEGG to evaluate the expression pattern on the basis of gene function and pathway. The result showed significantly changed genes were related to MAPK signaling pathway (Figure 5).

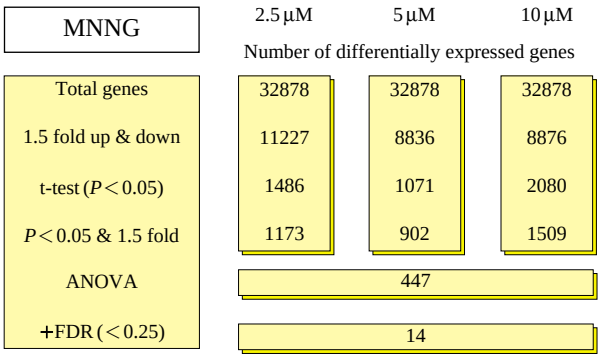

Figure 3. Microarray analysis results to identify differentially expressed gene of MNNG.

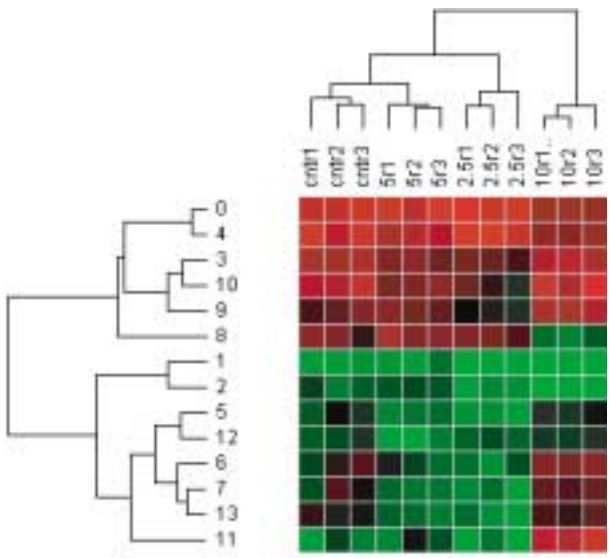

Figure 4. Hierarchical clustering analysis from >1.5 fold differentially changed genes (14 genes) in MNNG ($P < 0.05$).

In the case of H_2O_2, we identified a total of 188 genes with statistically significant changes using same analytical approach (Figures 6, 7, 8). A repesentative gene list of differentially-regulated genes is presented in Table 2. Altered genes included neuregulin 4, sulfiredoxin 1 homolog, endothelial adhesion molecule, and ATPase. With pathway analysis, folate synthesis pathway was related to H_2O_2 exposure.

Discussion

Genotoxic stress causes a variety of cellular responses including the transcriptional activation of genes regulating DNA repair, cell cycle arrest, and apoptosis[6-9]. Although all genotoxins produce such general responses, the mechanisms governing response to divergent forms of DNA damage are potentially diverse themselves. It is thus imperative that gene expression profiles be anchored to biological endpoints that can be quantified[10-12]. The cellular responses to genotoxic stress are partly mediated by the activation of signal transduction pathways involving mitogen-activated protein kinases (MAPKs), including the extracellullar signal-regulated kinases (ERKs), the c-Jun NH_2-terminal kinases (JNKs), and the p38 kinases.

N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), alkylating agent, is an environmental carcinogen that causes DNA lesions leading to cell death. MNNG and related genotoxic agents are extremely mutagenic, carcinogenic, and evoke a strong cell cycle arrest and /or apoptotic response. MNNG methylates various nucleophilic centers within the DNA molecule with methylation of the N3 position of adenine and the N7 and O6 position of guanine being the predominant lesions. N3MeA and N7MeG lesions are efficiently repaired by base excision repair (BER). However, the cytotoxic and mutagenic properties of MNNG are thought to principally stem from the methylation of

Table 1. Representative list of differentially expressed genes by MNNG in TK6 cells.

Gene bank accession No.	Gene name	Fold change		
		2.5 μM	5 μM	10 μM
AF119663	Guanine nucleotide binding protein, gamma 12	1.87	1.49	−1.31
AY422212	2-aminoadipic 6-semialdehyde dehydrogenase	−1.15	−1.06	1.63
BC000280	Myosin, heavy polypeptide 10, non-muscle	−1.08	1.30	1.93
AX878931	PAP associated domain containing 1	2.06	1.56	−1.02
AY459346	Phosphodiesterase 3B, cGMP-inhibited	2.32	1.88	−1.49
AB060283	Wingless-type MMTV integration site family, member 9A	1.94	1.47	−1.94
BC017303	Villin 1	1.00	−1.41	3.46
AK092507	Aldehyde dehydrogenase 7 family, member A1	1.13	1.96	−1.09

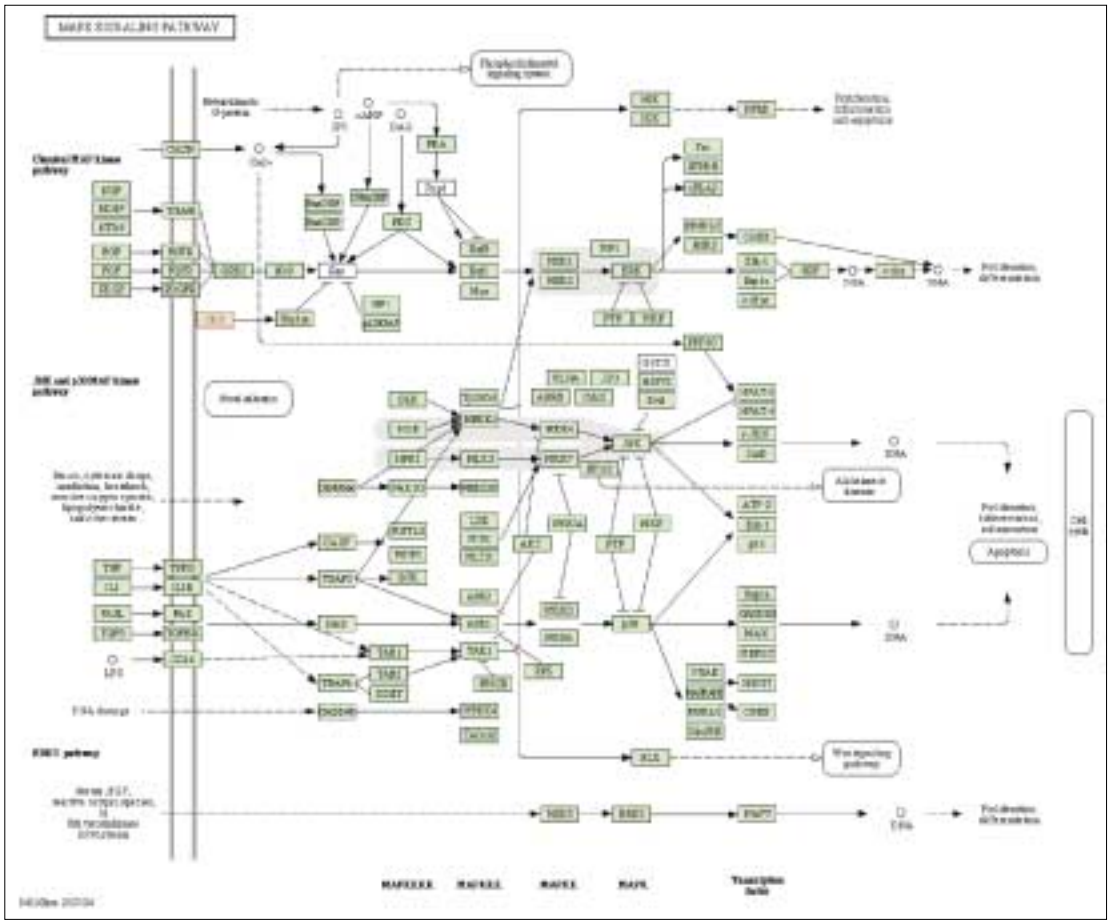

Figure 5. Gene ontology of differentially expressed genes by MNNG in TK6 cells.

H_2O_2	25 μM	50 μM	100 μM
	Number of differentially expressed		
Total genes	32878	32878	32878
1.5 fold up & down	13571	14821	9385
t-test ($P<0.05$)	5599	5084	1282
$P<0.05$ & 1.5 fold	5031	4710	1046
ANOVA	2557		
+FDR (<0.25)	188		

Figure 6. Microarray analysis results to identify differentially expressed gene of H_2O_2.

the O6 position of guanine (O6MeG)[13]. Direct repair of mutagenic O6MeG lesions is accomplished by the repair protein methylguanine-DNA methyltransferase (MGMT). Accordingly, lost or diminished MGMT activity results in MNNG-induced increased lesion load and sensitivity to MNNG[14].

Hydrogen peroxide (H_2O_2), free radical based DNA -damaging agent, mainly cause base substitution mutations. H_2O_2 is formed in the body by the conversion of superoxide anion radicals by superoxide dismutases. H_2O_2 subsequently undergoes a Fenton reaction to form the hydroxyl radical (· OH) which primarily cause double bond addition, but also abstract hydrogen and produce stand breaks[15].

We investigated the genotoxicant-induced changes in gene expression patterns as a function of toxicity and mutagenesis, using the TK6 human lymphoblast cell line as model system. To determine if the transcriptional responses of cells to toxicant exposures showed a dose response, we compared expression profiles at doses of the compounds that give different levels of toxicity and mutagenicity.

Analyzing gene expression, we identified a total of 447 genes with statistically significant changes in expression after MNNG treatment, and 2,557 genes whose expression levels were significantly changed

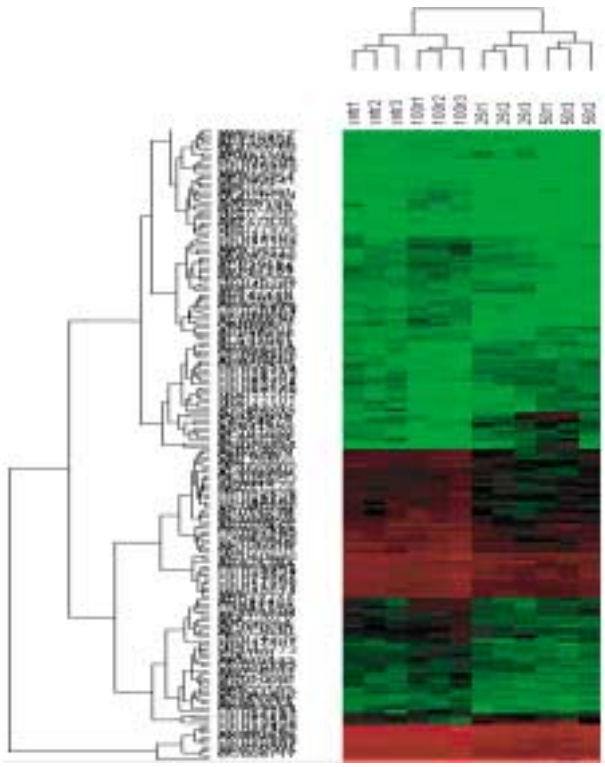

Figure 7. Hierarchical clustering analysis from >1.5 fold differentially changed genes (188 genes) in hydrogen peroxide ($P<0.05$).

by treatment with H_2O_2. These included guanine nucleotide binding protein, gamma12, 2-aminoadipic 6-semialdehyde dehydrogenase, aldehyde dehydrogenase 7 family, member A1, neuregulin 4, sulfiredoxin 1 homolog, endothelial adhesion molecule, and ATPase. (Table 1, 2). These genes function in cell survival, cell growth and hence their decreased expression could be indicators of toxicity. In addition, with low concentration of MNNG, Comet assay showed significant Comet tail formation, indicating that strand breaks are accumulated and MAPK signaling pathway was related to the MNNG-induced genotoxicity.

Torsten Dunkern *et al*. showed that activation of ATM/ATR by MNNG induced DNA damage leads to activation of p38 MAPK, which involves in the G1 checkpoint in mammalian cells[16]. It is widely believed that it is the presence of broken DNA strands that activates the catalytic activity of ATM. The ATM gene is mutated in ataxia-telangiectasia, a pleiotropic autosomal recessive disorder characterized by progressive cerebellar degeneration, oculocutaneous telangiectasia, immunodeficiency, cancer predisposition, and an extreme sensitivity to ionizing radiation (IR). Cells derived from ataxia-telangiectasia patients exhibit chromosomal instability and a profound defect in all cellular responses to DNA double strand breaks (DSBs). Downstream targets of ATM/ATR kinase activity that partially delineate DNA damage-activated cell cycle checkpoint signaling pathways have been recently reported. The best characterized checkpoint pathways involve p53, Chk1, Chk2, c-Abl, and BRCA1[17-24].

In summary, our data indicate that the value of the gene expression profiling technology is its potentials to provide mechanistic insight into the mode of action of a genotoxic compound. However, 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.

Methods

Materials

Male N-methyl-N′-nitro-N-nitrosoguanidine (MNNG) was purchased from TCI Co.(Japan). Hydrogen peroxide was purchased from Fluka. HEPES (N-[2-Hydroxylehtyl]piperazine-N′-[2-ethanesulfonic acid]), 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). Comet assay kit was purchased from Trevigen INC. (USA)

Cell Culture

Human lymphoblastoid line TK6 was derived from the parental human lymphoblastoid line HH4 and heterozygous for thymidine kinase (TK), an enzyme which phosphorylates thymidine and its toxic analogues in an ATP-dependent reaction. Cells were maintained in exponential growth by daily dilution in RPMI 1640 medium supplemented 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. Cell counts were taken daily using a Coulter Counter, and cultures were diluted to 5×10^5 cells/mL.

Cytotoxicity

Cytotoxicity was determined by MTS assay. Cells were treated with various concentrations of MNNG (0.006-0.75 mM) or hydrogen peroxide (0.01-5 mM)

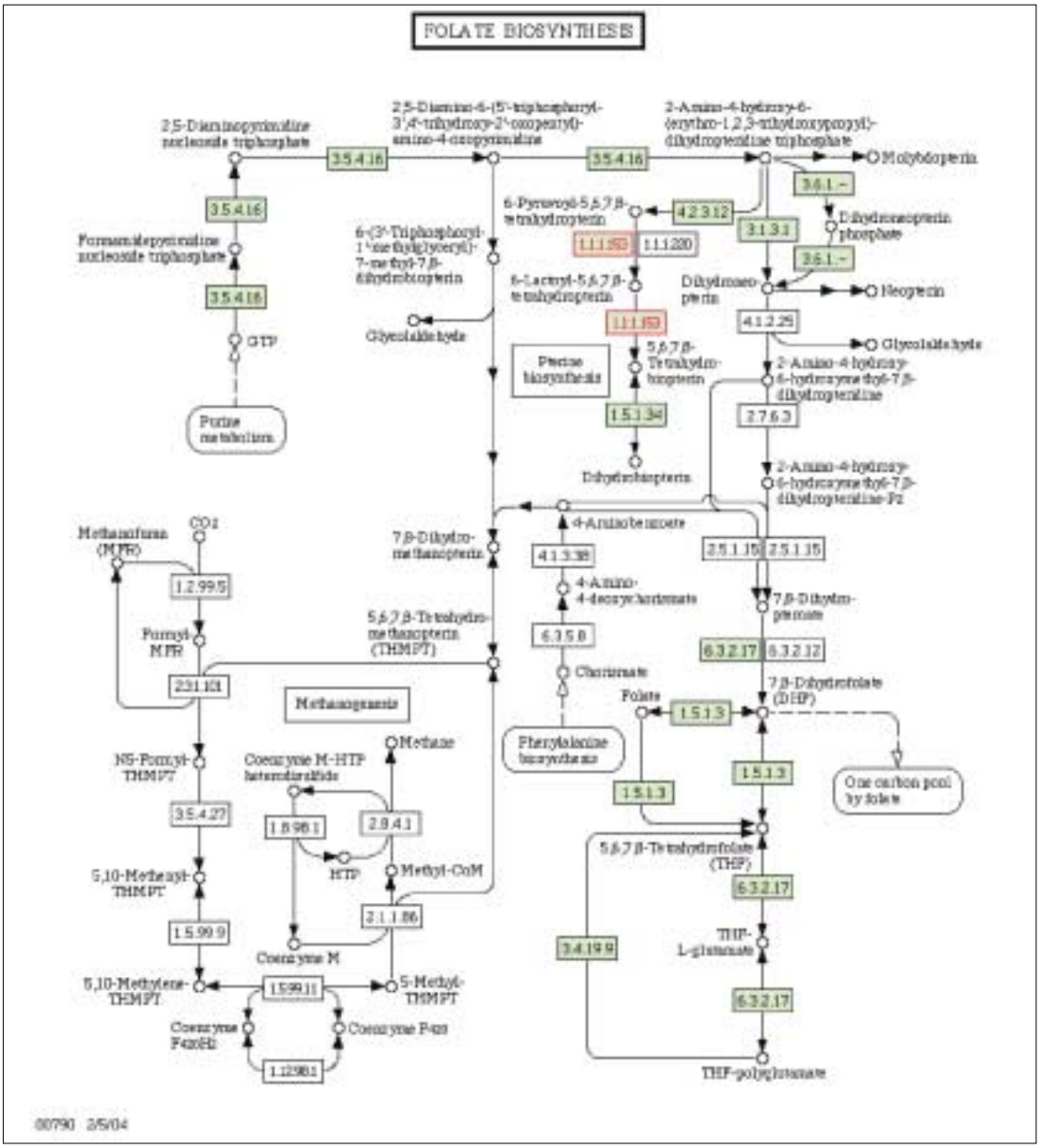

Figure 8. Gene ontology of differentially expressed genes by H_2O_2 in TK6 cells.

for 2 hr. After incubation of the cells for 2hr in test chemicals containing medium a sample of the culture was mixed with MTS (333 µg/mL) and PMS (25 µM) mixture solution. After 2 hr, O.D. was measured at 490 nm.

Comet Assay

The comet assay was performed according to recommendations published elsewhere[25]. Briefly, TK6 cells were grown at 37˚C in a 5% CO_2 incubation. Cells were seeded in 12 well plates (1×10^6 cells/mL) and were exposed to a different concentration of MNNG and H_2O_2 for 2 hr. 20 µL of cell suspension were mounted in 1% agarose on slide glass. Slides were immersed in a cold lysing solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, pH 10, 1% (v/v) Triton X-100 and 10% (v/v) DMSO) for 1.5 hr at 4˚C and then for 20 min in the electrophoresis buffer (0.3 M NaOH, 1 mM EDTA, pH > 13). Slides were electrophoresed and neutralized using Tris buffer (0.4 M Tris, pH 7.5)

Table 2. Representative list of differentially expressed genes by hydrogen peroxide in TK6 cells.

Gene bank accession No.	Gene name	Fold change		
		25 μM	50 μM	100 μM
AX878224	Chromosome 20 open reading frame 158;death associated transcription factor 1	−3.37	−3.21	1.53
106698	Peroxisome proliferative activated receptor, γ, coactivator 1, alpha	4.33	2.79	−1.18
AJ243670	NICE-4 protein	−3.15	−5.88	1.71
L48546	Tuberous sclerosis 2	−2.61	−3.79	1.9
AK027590	Death-associated protein kinase 3	−2.61	−3.55	1.75
AB055890	A kinase (PRKA) anchor protein 13	2.35	1.43	−1.11
BC035053	Ring finger protein 103	2.3	1.5	−1.12
AL133626	H2A histone family, member J	2.27	2.09	−1.15
BC072414	THAP domain containing 10	2.23	1.84	−1.48
AJ303119	Pleiomorphic adenoma gene-like 1	2.22	3.38	−1.09
AJ617628	Synaptotagmin XIV-like	2.18	2.02	−1.04
BC026125	Chaperonin containing TCP1, subunit 6B (zeta 2)	2.18	1.7	−1.2
BC064591	De-etiolated 1	2.16	1.9	−1.13
BC033789	Actin-like 7B	−2.16	1.58	−1.09
BC035625	Early growth response 2	2.05	1.29	−1.61
BC032645	Chromosome 10 open reading frame 88	2.07	1.87	−1.11
BC021901	RAB21, member RAS family	2.04	1.82	−1.01
BC031624	Retinitis pigmentosa GTPase regulator	2.01	2.63	−1.11
U60805	Oncostatin M receptor	1.98	−1.18	1
BC035494	Origin recognition complex, subunit 3-like (yeast)	1.91	1.69	−1.04
AB078417	Ras-induced senescence 1	−1.91	−1.62	2.09
AK097433	Acidic repeat containing	1.9	1.37	−2.3
BC004483	N-acetyltransferase 6	−1.82	−2.19	1.75
AL163301	Chromosome 21 ORF 70	−1.8	−4.36	1.68
AF114834	MAX dimerization protein 3	1.79	−1.61	1.18
BC047773	Bone morphogenetic protein receptor,type IB	1.77	2.17	−1.5
X55322	Neural cell adhesion molecule 1	1.77	−1.02	−1.7
BC015949	Leucine carboxyl methyltransferase 2	1.76	2.09	−1.11
AB022277	Tubulointerstitial nephritis antigen	1.76	1.24	−2.36
AJ006198	tyrosylprotein sulfotransferase 2	1.75	1.82	−1.7
AK131391	protein phosphatase 2, regulatory subunit B (B56), γ isoform	1.73	2.23	−1.01
AF040253	suppressor of Ty 5 homolog	1.73	−2.1	1.53
AL050258	tuftelin interacting protein 11	1.73	1.66	−1.09
BC034934	Myocardin-related transcription factor B	1.72	1.75	−1.08
BC051305	Transient receptor potential cation channel, subfamily V, member 2	1.7	−1.94	2.35
BC012629	Thyroid receptor interacting protein 15	1.68	2.04	−1.07
AK027360	G protein-coupled receptor 128	1.68	2.03	−1.21
AF208291	Homeodomain interacting protein kinase 2	1.68	1.06	−2.37
AL832891	Cysteine-rich, angiogenic inducer, 61	−1.67	−1.5	2.41
AK075109	Ceroid-lipofuscinosis, neuronal 5	1.67	1.38	−1.03
BX647796	Heart alpha-kinase	1.67	1.07	−1.86
AK056162	Jumonji domain containing 2D	1.66	2.6	−1.29
BC037574	Ubiquitin specific protease 46	−1.65	−1.67	1.66
AK057100	Sialytransferase 7 F	1.64	1.82	−1.19
AK074729	G protein-coupled receptor 19	1.63	1.05	−1.87
BC008717	Casein kinase 1, α 1	1.62	1.85	−1.01
BC002636	Zinc finger protein 495	1.61	1.41	−1.09
BC011731	Ras-GTPase activating protein SH3 domain- binding protein 2	1.6	2.00	−1.16
BC016697	Threonine synthase-like 1	1.59	1.54	−1.09
BC023576	Granulysin	1.58	−3.26	−1.63
BC017050	2, 3-Bisphosphoglycerate mutase	1.58	1.71	−1.13
BC038507	Glutaminase	1.57	1.66	−1.06

and stained with ethidium bromide (20 μg/mL). Cells were analyzed using a Comet Image Analysis System, Version 5.5 (Kinetic Imaging Ltd., Andor Bioimaging Division, Nottingham, UK).

RNA Preparation

At 2 hr post exposure, aliquots of cells were pelleted by cenrifugation 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 ratio was between 1.8 and 2.0 and 260/280 nm ratio between 2.0 and 2.2, respectively.

Microarray Analysis Using Applied Biosystems Array System

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 acqusition and analysis were performed using Applied Biosystems Chemiluminescene Detection Kit and Applied Biosystems 1700 Chemiluminescent Microarray Analyzer.

Differentially Expressed Genes Analysis

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 (base 2) and normalized so that median 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 each given time point. 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 each given time point. 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. Gene expression values were manipulated and visualized with the R-packages (Free Software under the terms of the Free Software Foundation's GNU General Public License). For analysis of data correlation, correspondence analysis (COA) was also performed using the AB1700 package in R. 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*-value < 0.05.

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