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Alteration in miRNA Expression Profiling with Response to Nonylphenol in Human Cell Lines

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Abstract

Exposures to environmental chemicals that mimic endogenous hormones are proposed for a number of adverse health effects, including infertility, abnormal prenatal and childhood development and above all cancers. In addition, recently miRNA (micro RNA) has been recognized to play an important role in various diseases and in cellular and molecular responses to toxicants. In this study, endocrine disrupting environmental toxicant, nonylphenol (NP) was treated to MCF-7 (Human breast cancer cell) and HepG2 (Human hepatocellular liver carcinoma) cell line at 3 hrs and 48 hrs time point and miRNA analysis using *mir*Vana™ miRNA bioarray was performed and compared with total mRNA microarray data for the same cell line and treatment. Robust data quality was achieved through the use of dye-swap. Analysis of microarray data identifies a total of 20 and 11 miRNA expressions at 3 hrs and 48 hrs exposure to NP in MCF-7 cell line and a total of 14 and 47 miRNA expression at 3 hrs and 48 hrs exposure respectively to NP in HepG2 cell line. Expression profiling of the selected miRNA (let-7c, miR-16, miR-195, miR-200b, miR200c, miR-205, and miR-589) reveals changes in the expression of target genes related to metabolism, immune response, apoptosis, and cell differentiation. The present study can be informative and helpful to understand the role of miRNA in molecular mechanism of chemical toxicity and their influence on hormone dependent disease. Also this study may prove to be a valuable tool for screening potential estrogen mimicking pollutants in the environment.

Keywords: Expression profiling, Human cell line, miRNA, Endocrine disrupting toxicant, Nonylphenol (NP), miRNA bioarray

The study of gene expression changes due to environment-induced toxicity, with emphasis on determination of corresponding gene functions, pathways, and regulatory networks, are new research field of toxicogenomics. One such environmental toxicant, nonylphenol (NP) is an industrially synthesized product which is used as a chemical intermediate for antioxidants for rubber and plastics, phenolic resins, nonionic and anionic surfactants and polyvinyl chloride plasticizers. Such polyphenols and phenolic metabolites of environmental agents have been identified as potential agent for altering endocrine function thereby producing adverse health effects in humans and other species. NP has been shown to have estrogenic properties and this estrogen-like activity of NP was observed to induce cell proliferation in human estrogen-sensitive MCF7 breast cancer cell[1]. NP is also found in some food products, cosmetics, and drinking water. NP has been detected in drinking water[2], and has been reported to leach from tubing for milk processing and from plastics used in food packaging[3]. In 1991, Soto and her co-workers described the estrogenic activity of NP leached from a modified polystyrene plastic used in production of laboratory plasticware. Recent studies have demonstrated that para-NP is found on most of our fruits and vegetables, probably because of the widespread use of NP as a carrier in pesticide formulations[4]. NP has also shown to over-express gene related to cell proliferation, apoptosis and MARK signaling pathway in HK-2 cell line[5].

The key component of recent toxicology is the study of allostatic gene expression, which is defined as drug or environment induced changes in gene expression that is indicative of disease and/or pharmacological or environmental exposure. It is proposed that expressions of more than one third of human genes are under

miRNA control; which explains wide action of miRNA in disease and drug response[6]. miRNAs are short (18-26 nucleotides) non-coding RNA molecules, that binds to messenger RNA and thereby regulate the translation or degradation of it[7,8]. In mammals, miRNA negatively regulate their targets by either binding to imperfect complementary sites within the 3'-untranslated regions of their mRNA targets[7] reducing protein level of target gene or by targeting specific cleavage of homologous mRNAs[9] inducing degradation of target mRNAs. miRNA is reported to be involved in estrogen regulated physiological process and disease[10]. miRNA expression can be controlled by various chemotherapeutics and chemicals such as ethanol in rodents and humans[11-15]. Xenobiotic-mediated miRNA expression has been directly linked with downstream protein expression and cell proliferation in mice[16]. These studies demonstrate the potential regulatory functions of miRNAs in different cells and tissues.

In this study we provide information of how human cell line responds to environmental EDCs. Human breast cancer cell line MCF-7 and Human hepatocellular liver carcinoma cell line HepG2 was treated with nonylphenol at different time point and mRNA expression profiling was carried out using the Nimblegen 4 plex human whole genome array, which contains approximately 24,000 human genes and miRNA expression is analyzed by *mir*Vana™ miRNA bioarray.

Cytotoxicity Assay of Nonylphenol in Cell Lines

MTT assay is carried out to determine the cell viability of MCF-7 and HepG2 cell line after exposure to NP at various concentrations. Based on the results of MTT assay, the 20% cell viability inhibition concentration (IC_{20}) of NP in each cell line is calculated from the linear dose-dependant curves as shown in the Figure 1. As a result, the IC_{20} value of NP in MCF-7 and HepG2 cell line are found to be 12 μM and 52 μM respectively.

Gene Expression Data Analysis

Total RNA isolated from MCF-7 and HepG2 cell line treated with 12 μM and 52 μM nonylphenol respectively were subjected to microarray analysis. 2 fold gene expression changed were analyzed by comparing treated with control group in both time point. A total of 389 and 857 genes were differentially expressed in MCF-7 cell line at 3 hrs and 48 hrs exposure to NP respectively compared to 1,358 and 4,313 genes expressed in HepG2 cell line at 3 hrs and 48 hrs exposure to NP respectively. Table 1 shows the number of gene up-regulated and down regulated in this two cell line. Hierarchical clustering was implemented for the visualization and biological interpretation of the gene expression pattern. In this clustering analysis, the pattern of gene expressed in both the cell line shows

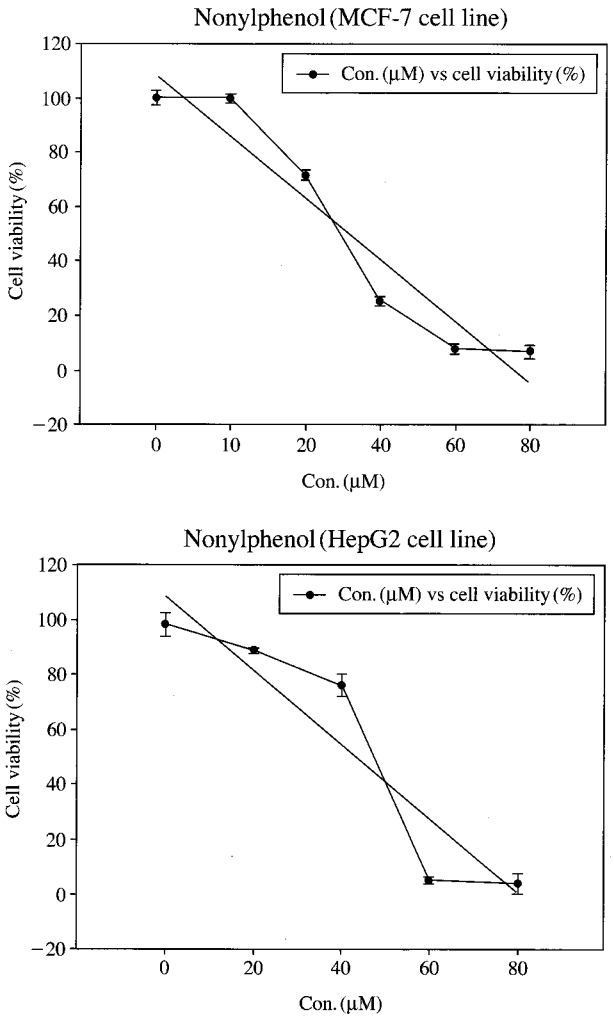

Figure 1. Cell viability after the exposure with nonylphenol was analyzed by the MTT assay. The change in cell death is shown as the % of control. 20% inhibition concentration (IC_{20}) values of nonylphenol are calculated as 12 μM and 52 μM for MCF-7 and HepG2 cell line respectively.

Table 1. 2 folds gene regulation in each cell line with nonylphenol.

Sample name	Treatment/ Time	2 fold up regulated genes (>2.0)	2 fold down regulated genes (<0.5)	Total
MCF-7 cell line	12 μM/3 hrs	140	249	389
	12 μM/48 hrs	374	483	857
HepG2 cell line	52 μM/3 hrs	620	738	1,358
	52 μM/48 hrs	2,141	2,172	4,313

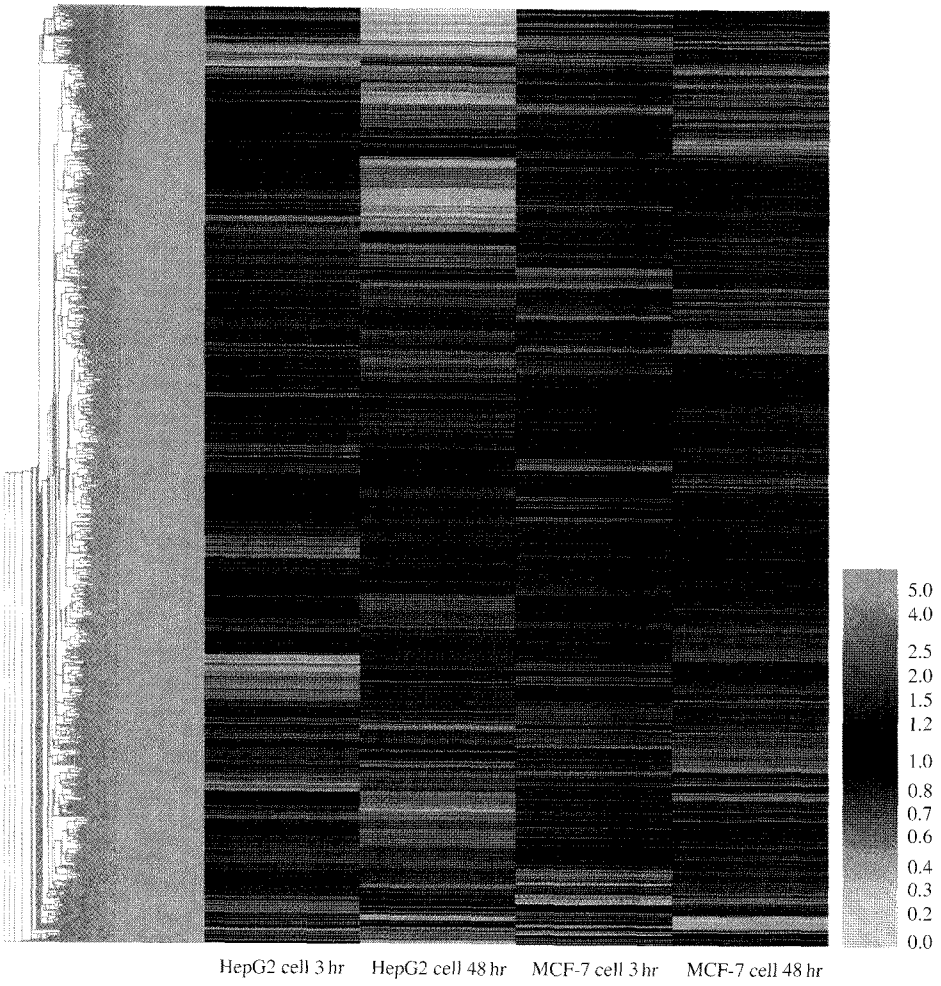

Figure 2. Hierarchical cluster image showing the differential gene expression profiles in cells by nonylphenol treatment. Rows represent genes and columns represent samples. Red and green colors represent up-regulation and down-regulation respectively and black color represent no change.

Table 2. 2 folds miRNA expression in each cell line in response to nonylphenol.

Sample name	Treatment/ Time	Up (>2.0)	Down (<0.5)	Total
MCF-7 cell line	12 μM/3 hrs	19	1	20
	12 μM/48 hrs	2	9	11
HepG2 cell line	52 μM/3 hrs	0	14	14
	52 μM/48 hrs	9	38	47

results that are at variance with respect to NP exposure (Figure 2).

miRNA Expression Data Analysis

miRNA microarrays were used to analyze the miRNA expression profiles in MCF-7 and HepG2 cell line exposed to NP. We identified a total of 20 and 11 miRNA expressed respectively in MCF-7 3 hrs and MCF-7 48 hrs exposure to NP as a result of 2 fold changes. Similar analysis resulted expression of 14 and 47 miRNA at 3 hrs and 48 hrs respectively in HepG2 cell line. Table 2 provide the number of up-regulated and down regulated miRNAs as a result of 2 fold change. The result shows decrease in miRNA expression with time in MCF-7 cell line whereas there is a 3 times increase of miRNA expression with time in HepG2 cell line. The miRNAs expressed in MCF-7 cell line at 3 hrs are very different than the miRNAs expressed at 48 hrs. The same is true for the miRNA expression for 3 hrs and 48 hrs in HepG2 cell line (Figure 4).

Functional Analysis

A functional analysis of genes showing meaningful expression patterns in each experiment was also conducted. Genes that evidenced expressional changes of over 2 fold as compared to the comparison groups in the functional analysis were analyzed using a gene ontology database. In McF-7 cell line, as a result of time dependent exposure to NP, the function of gene expressed is found to be very similar relating to alco-

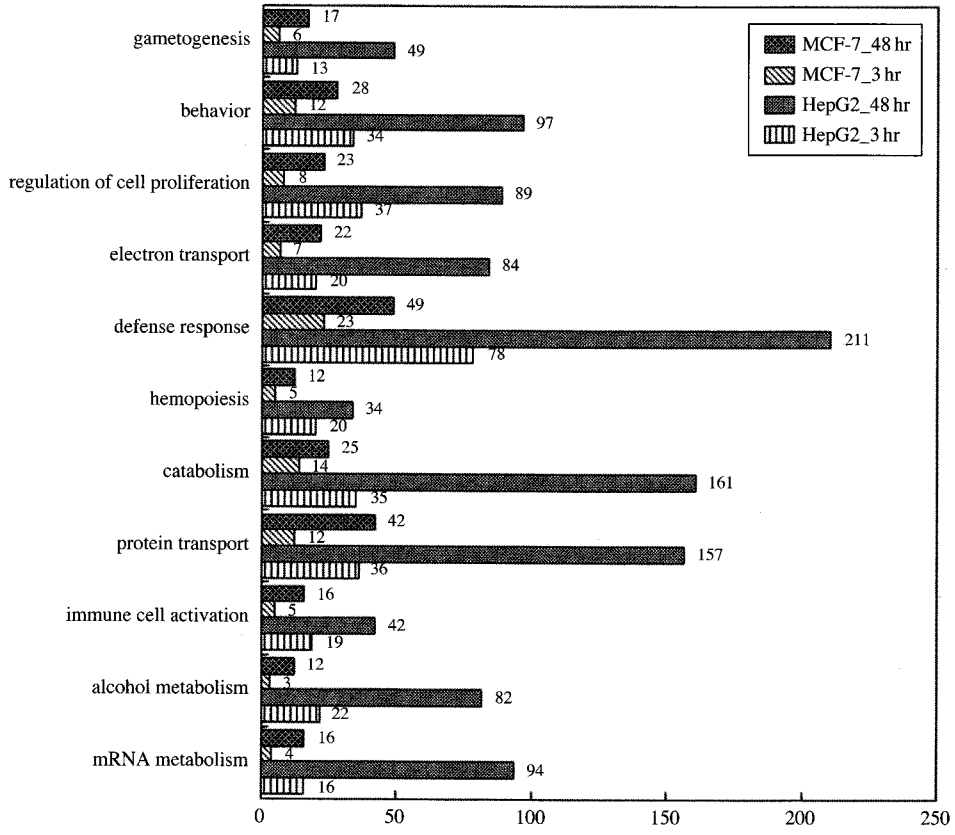

Figure 3. Functional classification of expressed gene in cell lines with response to nonylphenol exposure.

Table 3. Functional classification of expressed gene.

Function	MCF-7_3 hr	MCF-7_48 hr	HepG2_3 hr	HepG2_48 hr
mRNA metabolism	4	16	16	94
alcohol metabolism	3	12	22	82
immune cell activation	5	16	19	42
protein transport	12	42	36	157
catabolism	14	25	35	161
hemopoiesis	5	12	20	34
defense response	23	49	78	211
electron transport	7	22	20	84
regulation of cell proliferation	8	23	37	89
behavior	12	28	34	97
gametogenesis	6	17	13	49

hol metabolism, defense response, regulation of cell proliferation, immune cell activation, mRNA metabolism and gametogenesis (Figure 3). Similarly functional analysis of gene expressed in HepG2 cell line in response to NP exposure at two time point classifies function relating to alcohol metabolism, defense response, regulation of cell proliferation, immune cell activation, mRNA metabolism and gametogenesis. Expression of gene with respective function is much higher in HepG2 cell line compared to MCF-7 cell line and also varies at two time point (Table 3).

In view of the fact that miRNA negatively regulate their target gene, up-regulation of miRNA-let-7c in MCF-7 cell line evidenced down regulation of target gene associated with anti-apoptosis, regulation of apoptosis, cell differentiation, regulation of transcription, regulation of angiogenesis and DNA replication (Table 4). Co-activation of up-regulated miRNAs-miR16 and miR-195 shows down regulation of gene related to lipid metabolism, immune response, anti-

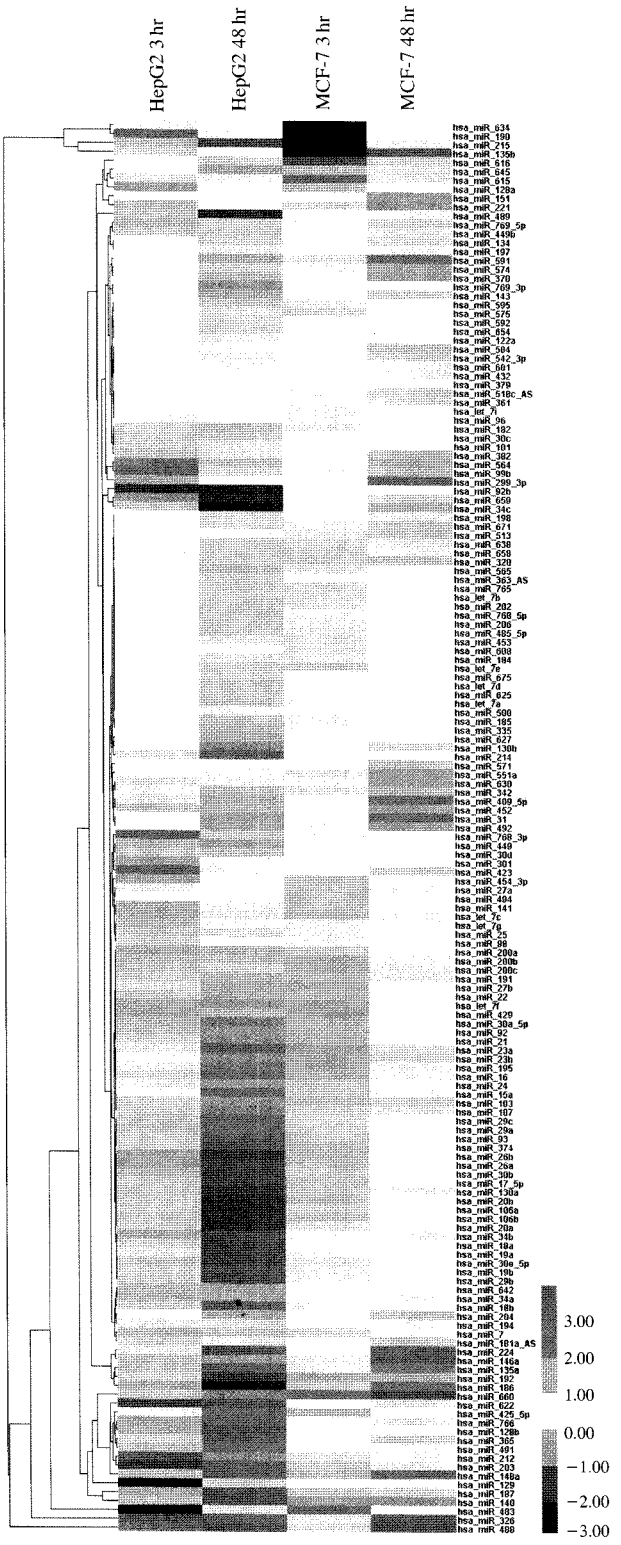

Figure 4. Clustering analysis of the expression of human miRNA in two cell lines with response to nonylphenol treatment. The expression level is defined by the color key on the right side of the figure.

apoptosis and co activation of up-regulated miR-200b and miR-200c down-regulates genes with lipid metabolism, immune response, anti-apoptosis, sensory perception and regulation of transcription functions. In HepG2 cell line, up-regulation of miRNAs-miR205 and miR-589 resulted in the down regulation of target gene related to immune response, inflammatory response, response to stimulus, spermatogenesis, cell differentiation, sperm motility and intracellular signaling cascade (Table 4).

Discussion

In this report we describe nonylphenol-induced expression changes in MCF-7 and HepG2 cell line with respect to miRNAs regulation. Combining the miRNA expression data with the translational data will help to identify potential miRNA-mRNA interaction. Large-scale microarray analyses that compare changes in gene expression in response to the presence of specific miRNAs show that many targets are subject to down-regulation at the mRNA level[17]. In our microarray experiment we detected up-regulation of miRNAs involved in down regulation of its target genes with response to NP.

In MCF-7 cell line, up-regulation of miRNA, let-7c at 3 hrs NP exposure down regulated genes (ALOX12, CHD4, CIZ1, COL4A2, GNAL, and SEMA4C) related to anti-apoptosis, regulation of apoptosis, cell differentiation, regulation of transcription, regulation of angiogenesis and signal transduction. Also co-actively up-regulation of miR-16 & miR-195 and miR200b & miR-200c down-regulated target gene, ACOT7, ALOX12, PLUNC and ACOT7, IL24, WBSCR22, WDR1, NRBF2 respectively and are related to cellular function such as lipid metabolism, immune response, anti-apoptosis, sensory perception and regulation of transcription. This reflects involvement of miRNA in regulating cellular and physiological process associated with effect of hormone[10] like EDCs. It is also observed that intensity of down-regulated target gene at 3 hrs NP exposure tends towards up-regulation with respect to long time exposure to NP at 48 hrs (Table 4). In mammal, changes in miRNA expression were also observed with exposure to toxicant[18].

MCF-7 cell line exposed to NP for 48 hours shows up-regulation of a different set of miRNA (miR-205 and miR-589) with down-regulated target genes TBX21, GRK7, NHLH1, DNAH9, XCR1, ATP1A4 by miR-205 related to regulation of immune response, inflammatory response, response to stimulus, spermatogenesis, cell differentiation and sperm motility where-

Table 4. List of 2 fold up-regulated miRNA with its 2 fold down-regulated target genes.

miRNA	Target gene	MCF-7 3 hrs	MCF-7 48 hrs	Function
hsa-let-7c	ALOX12	0.463	0.857	anti-apoptosis
	CHD4	0.461	0.943	transcription factor binding
	CIZ1	0.395	0.869	regulation of progression through cell cycle
	COL4A2	0.366	0.536	regulation of angiogenesis
	GDF5	0.487	0.808	regulation of apoptosis
	GNAL	0.451	0.969	signal transduction
	KTN1	0.437	0.565	behavior
	PADI4	0.483	0.579	regulation of transcription
	SEMA4C	0.500	1.609	cell differentiation
	TK1	0.361	1.367	DNA replication
hsa-miR-16 & hsa-miR-195	ACOT7	0.422	0.734	lipid metabolism
	AGPAT6	0.496	0.840	regulation of body size
	ALOX12	0.463	0.857	anti-apoptosis
	PLUNC	0.423	1.528	innate immune response
hsa-miR-200b & hsa-miR-200c	ACOT7	0.422	0.734	lipid metabolism
	CCDC50	0.432	1.181	sensory perception
	CDX4	0.249	0.751	regulation of transcription
	CMPK	0.209	0.931	nucleic acid metabolic process
	IL24	0.417	1.037	apoptosis; immune response
	NRBF2	0.346	0.621	regulation of transcription
	WBSCR22	0.472	0.965	metabolic process
	WDR1	0.482	1.220	sensory perception
hsa-miR-205	TBX21	0.665	0.499	regulation of immune response
	GRK7	1.564	0.499	response to stimulus
	INTS5	0.661	0.494	snRNA processing
	NHLH1	0.667	0.465	cell differentiation
	HECW1	0.779	0.435	protein modification
	ERGIC1	1.558	0.431	transport
	DNAH9	1.044	0.430	spermatogenesis
	SH3BP2	0.682	0.404	intracellular signaling cascade
	XCR1	0.606	0.371	inflammatory response
	ATP1A4	1.219	0.352	sperm motility
	ATP6V1B1	0.669	0.329	excretion; ossification
hsa-miR-589	C1QC	0.950	0.441	immune response
	PIK3R3	1.019	0.439	intracellular signaling cascade
	PACS1	0.649	0.432	protein targeting to Golgi
	SPTB	0.820	0.425	barbed-end actin filament capping
	PAN3	0.656	0.310	mRNA catabolic process
	TBXAS1	0.776	0.299	blood coagulation

as miR-589 up-regulated genes C1QC, PIK3R3, PAN3, TBXAS1 with cellular function as immune response, intracellular signaling cascade, mRNA catabolic process and blood coagulation. As the effect of NP has been reported in many research to have character like EDCs[19], our experiment reflecting down-regulation of gene related to spermatogenesis and sperm motility supports the toxicity effect of NP and the possible use of microarray expression profile for the study miRNA/mRNA interaction. The miRNA expressed in MCF-7 cell line and HepG2 cell lines are very different in both the cell line which shows tissue specific expression of miRNAs[20,21]. These results indicate that the patterns and levels of miRNA expression are different in diverse cells and tissues, suggesting that they might have important roles in maintaining tissue and cell identity or function.

Also the pattern of miRNA expression varies in the same cell line at different time point in both MCF-7 and HepG2 cell line. This implies that the time of exposure to NP may be a crucial factor in determining effect of expression of miRNA on its target gene.

In conclusion, this report will give a new insight into the mechanism by which cells alter their translational profiles in response to toxicity and will highlight fundamental roles of miRNA species in the control of such altered translational events in toxicology.

Material & Methods

Chemical

Nonylphenol (NP) was purchased from Sigma-Aldrich in liquid form and stock solution is prepared with DMSO (cat.no.D-2438, 50 mL) purchased from Sigma-Aldrich.

Cell Culture Maintenance and Growth

Human breast cancer cell line (MCF-7) and Human Hepatocellular liver carcinoma cell line (HepG2) were purchased from Korea cell line Bank (South Korea). MCF-7 cell was cultured in Dulbecco's Modified Eagle Medium (DMEM; invitrogen, Gibco, low glucose) supplemented with 10% Fetal Bovine Serum (FBS; invitrogen, Gibco) and 1% Penicillin-Streptomycin (invitrogen, Gibco). HepG2 cell was cultured in Minimum Essential medium (MEM; invitrogen, Gibco) supplemented with 10% Fetal Bovine Serum (FBS; invitrogen, Gibco) and 1% Penicillin-Streptomycin (invitrogen, Gibco). Cells were maintained in a 37°C humidified incubator containing 5% CO_2 and were subcultured at an interval of 48-72 hours in 100 mm Petri-plates (Nunc; Paisley, UK) depending upon the cell state.

Cytotoxicity Test for Cell Viability

The cytotoxic effects of NP in MCF-7 and HepG2 cells were measured using an 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a tetrazole (MTT) assay, which is based on the ability of live cells to convert a tetrazolium salt into purple formazan. MCF-7 and HepG2 cells were seeded at a density of 2×10^4 cells/mL and 4×10^4 cells/mL respectively in 24-well microplates and incubated overnight. Cells were then exposed to various concentrations of nonylphenol in culture media and incubated at 37°C for a 48 hrs exposure time. After the incubation time MTT solution (4 mg/mL in PBS) is added to each well and incubated for 3 hrs. Cells were resuspended with DMSO (cat.no.D-2650, 100 mL, Sigma-Aldrich) and transferred to 96 well plates. The OD of purple formazan was measured at a wavelength of 540 nm. The IC_{20} cell proliferation was defined as the concentration that causes a 20% reduction in cell viability versus the solvent treated control.

Chemical Treatments and RNA Isolation

MCF-7 and HepG2 were seeded at a density of 6×10^6 cells/mL and 4×10^6 cells/mL in 100 mm Petri-plates and incubated at 37°C, 5% CO_2 for 24 hrs. Cells were then subjected to nonylphenol (IC_{20} for MCF-7 = 12 μM & HepG2=52 μM) exposure for 3 hr and 48 hrs. Following the respective incubation period, cells were washed with PBS and per plate are collected with 1 mL TRIzol Reagent™ (Invitrogen; Carlsbad, CA, USA).

Total RNA was extracted according to the TRI-REAGENT (MRC, OH) manufacturer's instructions. RNA concentration was quantified using NanoDrop and RNA quality was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies, Inc., CA).

Preparation of Fluorescent Probe and Hybridization

Total RNA sample (20 μg) was labeled with Cyanine (CY3) or Cyanine (Cy5) conjugated dCTP (Amersharm, Piscataway, NJ) via a reverse transcription reaction using reverse transcriptase, SuperScrip II (Invitrogen, Carlsbad, California). The labeled cDNA mixture are placed on Human Nimblegen 4-plex whole genome array (Roche, Nimblegen) and covered by Nimblechip X4 Mixer (Roche, Nimblegen). The slides were hybridized for 12 hr at 42°C in the MAUI system (Biomicro systems, Inc. UT). The hybridized slides were washed in 2 × SSC, 0.1% SDS for 5 min, 1 × SSC for 5 min, and 0.2 × SSC for 5 min at room temperature. The slides were then centrifuged at 3,000 rpm for 20 sec to dry.

40 μg of total RNA containing microRNA were labeled using *mir*Vana™ miRNA labeling kit (Ambion, Inc.) using CyDye (Amersham Biosciences) and hybridized according to Ambion *mir*Vana™ miRNA bioarrays version 9.2 hybridization protocol. The hybridized slides were incubated for 12 hr at 42°C in the MAUI system (Biomicro systems, Inc. UT). Dye-swap approach was employed to control for the gene-specific dye bias. Dye bias is defined as an intensity difference between samples labeled with different dyes attributable to the dyes instead of the gene expression in the samples.

Microarray Data Analysis

We used to NimbleGen Homo sapiens 4-plex array for gene expression profiles. The NimbleGen Homo sapiens 4-plex array was submitted to Roche NimbleGen Inc. for microarray design and manufacture using maskless, digital micromirror technology. Four replicates of the genome were included per chip. An average of 3 different 60-base oligonucleotides (60-mer probes) represented each gene in the genome. The arrays were analyzed using an Axon GenePix 4000B scanner with associated software (Molecular Devices Corp., Sunnyvale, CA). Gene expression levels were calculated with NimbeScan Version 2.4 (Roche NimbleGen, Inc., WI). Relative signal intensities for each gene were generated using the Robust Multi-Array

Average algorithm. The data were processed based on quantile normalization method using the NimbleScan Version 2.4 (Roche NimbleGen, Inc., WI). This normalization method aims to make the distribution of intensities for each array in a set of arrays the same. Data of miRNA expression were analyzed using GeneSpring GX 7.3.1 (Agilent technologies, CA) and background-adjusted, normalized, and log transformed intensity values were analyzed. Fold change filters included the requirement that the genes be present in at least 200% of controls for up-regulated genes and lower than 50% of controls for down-regulated genes. Hierarchical clustering data were clustered groups that behave similarly across experiments using GeneSpring GX 7.3.1 (Agilent technologies, CA).

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