

# Comparative Analysis of Gene Expression Patterns after Exposure to Nonylphenol in Human Cell Lines

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## Abstract

Nonylphenol (NP), a byproduct of industrial synthesis, is quite similar to estrogen in structure, and is known as an environmental estrogen that induces estrogenic disturbances. It has been utilized in several industries for the manufacture of skin cleaning materials, kitchen detergents, cosmetics, fabric detergents, and ink binder. Due to its characteristic strong estrogenic potency, NP is capable of disrupting the reproductive hormone system. Exposure to NP for a prolonged period increases the chances of developing breast and lung cancers. In this study, we conducted a comparative study of expression profiles between HK (Human Kidney cell), MCF-7 (Human breast cancer cell), and LNCaP (Human prostate cancer cell) cells treated with NP at the value of IC<sub>20</sub>, using Agilent whole genome microarrays. After comparative analysis, we detected some specific expression patterns in each of the cell lines. However, the expression patterns from the HK-2 and MCF-7 cells are quite similar. Interestingly, estrogen receptor 1 and 2 genes were downregulated only in

MCF-7 cells, whereas the androgen receptor (AR) gene evidenced overexpression in all 3 of the cell lines. The PDZK1 gene, which has been identified as an estrogen-responsive gene, has also been shown to be overexpressed in all 3 of the tested cell lines. The majority of differentially expressed genes in NP treatment were shown to be involved in cell proliferation, transcription, and signaling. These results may establish a framework for understanding the mechanisms underlying the toxicity of NP.

**Keywords:** Toxicogenomics, EDCs (Endocrine Disrupting Chemicals), Whole genome microarray, HK (Human Kidney Cell) cell, LNCaP (Prostate cancer cell), MCF (Human Breast Cancer Cell)

## Introduction

Nonylphenol, a typical example of Endocrine Disrupting Chemicals (EDCs), has been demonstrated to act in a fashion similar to that of normal hormones within the living body, as is also the case with DES (diethylstilbestrol), a synthetic estrogen, and another industrial chemical, bisphenol A. Currently, many synthetic and natural plant products, many of which are used extensively in a wide range of human endeavors, are suspected to be EDCs and are generally considered hazardous to humans<sup>1-3</sup>. These synthetic and natural plant materials are estimated to adversely affect not only humans, but also wild animals such as fishes, birds, amphibians, reptiles, and mammals. From this perspective, recent studies have shown that exposure to EDCs has immediate as well as long term effects on human health, including a reduction in sperm counts in males. Nonylphenol (NP) is the most abundant of these products, and accounts for 80% of the alkylphenol in the environment attributable to commercial sources; human exposure can occur through contaminated water, food, and skin contact. Additionally, NP has been reported to possess estrogenic properties in *in vitro* and *in vivo* assay systems, and to stimulate the proliferation of MCF-7 (Human breast cancer cell) cells, an estrogen-dependent human breast cell line. NP has also been demonstrated to exert a stimulating effect on epithelial cells of the Noble rat mammary gland, as well as uterotrophic effects in mice and rats. Alkylphenols are considered to interfere with the binding of estrogen to its recep-

tor. Several studies have reported on the adverse effects of NP on the development of the male reproductive tract. These effects include reduced testes size, decreased sperm production, and reduced reproductive organ weights. The results of a recent study have shown that not only the estrogen receptor, but also the androgen receptor can function as an antagonist. NP evidenced relatively high binding affinities for both AR and  $Er\alpha$  (estrogen receptor). NP may also function as an AR antagonist by influencing AR function; it inhibits the interaction of AR with its co-activator, androgen binding of AR nuclear translocation, and androgen-induced AR transcriptional activity<sup>7-11</sup>.

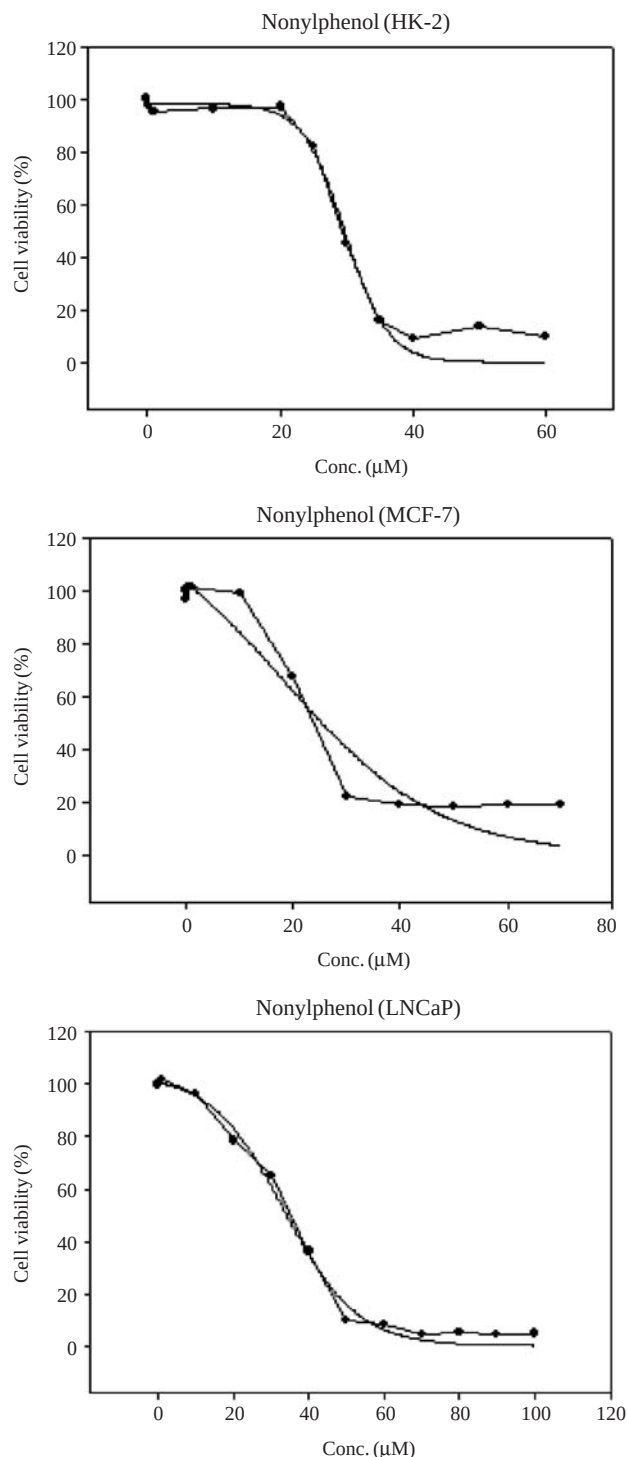
EDCs occurring at very low levels of density within the human body are quite difficult to trace, and it is necessary to verify the viability of new experimental methods continuously at the molecular level, as well as environment residual status investigations and accumulation status investigations, in order for the miniscule amount of materials present in the body to be accurately measured and analyzed. Expression profiling is one of the most advanced transcriptomic techniques used for the monitoring and evaluation of the effects of chemicals, and a microarray assay for the toxicogenomic evaluation of chemicals has been developed as a high-throughput and time/cost-saving system. Several assay methods are available for the evaluation of physiological effects of estrogenic chemicals via gene expression profiling in human cultured cells or animals using genome-wide microarrays<sup>12,13</sup>.

In this study, we assessed the differential expression of genes in HK (Human Kidney Cell), MCF-7 (Human Breast cancer cell: estrogen receptor (+)), and LNCaP (Human Prostate cancer cell) cell lines treated with nonylphenol, at various concentrations and different exposure times using a 44 k Agilent whole genome chip.

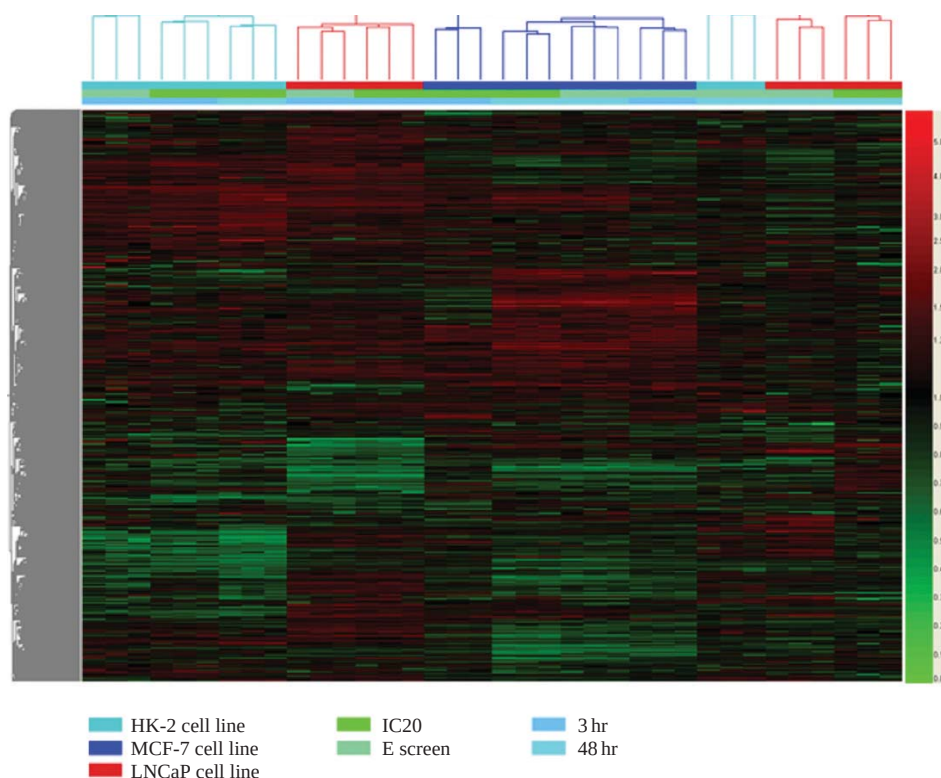
## Results

### Cytotoxicity of Nonylphenol in Cell Lines

The cell viability of HK, MCF-7, and LNCaP Cells after exposure to a range of concentrations of NP was determined via MTT assay. On the basis of the results of our MTT assay, the 20% cell viability inhibitory concentration ( $IC_{20}$ ) was calculated. Dose-dependent cell viability curves were obtained for each 3 h and 48 h of exposure to nonylphenol in human cells, as is shown in Figure 1. Examining the cell toxicity of nonylphenol in HK, MCF-7, and LNCaP, the density ( $IC_{20}$ ) results showing 20% of survival ratio



**Figure 1.** Cell viability after nonylphenol exposure was analyzed via an MTT assay. The reaction was halted by adding DMSO and the absorbance (optical density) was determined at 540 nm. The change in absorbance, which indicates the change in cell death, is expressed as the % of control. 20% inhibition concentration ( $IC_{20}$ ) values of NP were calculated as (a) HK  $IC_{20}$ : 2.5  $\mu$ M and (b) MCF-7  $IC_{20}$ : 12  $\mu$ M. (C) LNCaP:  $IC_{20}$  21  $\mu$ M.



**Figure 2.** Hierarchical cluster image showing the differential gene expression profiles between Nonylphenol treatments in cells.

**Table 1.** Differentially expressed genes in each cell line.

| Sample name     | Expressed genes  | 2 fold changed genes |             |
|-----------------|------------------|----------------------|-------------|
|                 |                  | Up (>2.0)            | Down (<0.5) |
| HK-2 cell line  | 1 $\mu$ M-3 hr   | 18770/33133          | 175         |
|                 | 1 $\mu$ M-48 hr  | 19433/33133          | 62          |
|                 | 25 $\mu$ M-3 hr  | 22510/33133          | 90          |
|                 | 25 $\mu$ M-48 hr | 22957/33133          | 499         |
| MCF-7 cell line | 1 $\mu$ M-3 hr   | 26629/33133          | 666         |
|                 | 1 $\mu$ M-48 hr  | 24533/33133          | 518         |
|                 | 25 $\mu$ M-3 hr  | 27429/33133          | 242         |
|                 | 25 $\mu$ M-48 hr | 27506/33133          | 672         |
| LNCaP cell line | 1 $\mu$ M-3 hr   | 24381/33133          | 139         |
|                 | 1 $\mu$ M-48 hr  | 25806/33133          | 354         |
|                 | 25 $\mu$ M-3 hr  | 24230/33133          | 84          |
|                 | 25 $\mu$ M-48 hr | 26280/33133          | 153         |

were 2.5  $\mu$ M, 12  $\mu$ M, and 21  $\mu$ M, respectively (Figure 1).

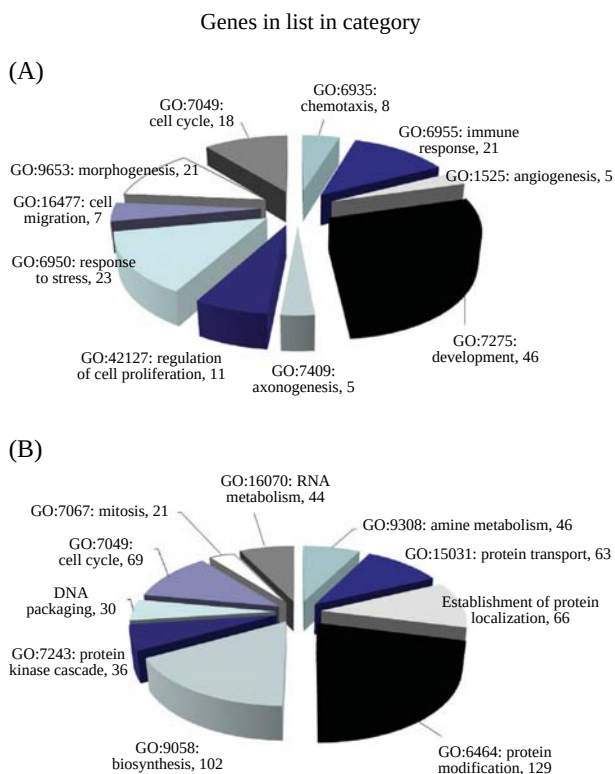
### Gene Expression Analysis

According to our results, gene expressions were assessed according to the cell lines used, and while both the HK-2 and MCF-7 cells evidenced similar expression patterns, the LNCaP cells, which are estro-

gen receptor (–), evidenced a distinct expression pattern. In the LNCaP cell line, expression was downregulated at early stages (3 hr), but evidenced little change in expression during late stages of the assay (48 hr). Both Estrogen receptor 1 and 2 genes were downregulated only in the MCF-7 cells, and the expression of the androgen receptor was increased in all three sample cell types. Because nonylphenol functions as an androgen receptor antagonist, cell proliferation and cell growth regulation were observed. The PDZK1 gene, which is generally recognized as an Estrogen-responsive gene, evidenced overexpression, just as has been reported in many previous studies (Figure 2). Additionally, 22,000 other genes evidenced expressional changes. Table 1 shows the genes whose expression patterns are regularly altered by NP in each of the cell lines, and provides a list of genes that were upregulated or downregulated by >2.0 fold, as shown by microarray.

### 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 the HK-2 cell line, as a result

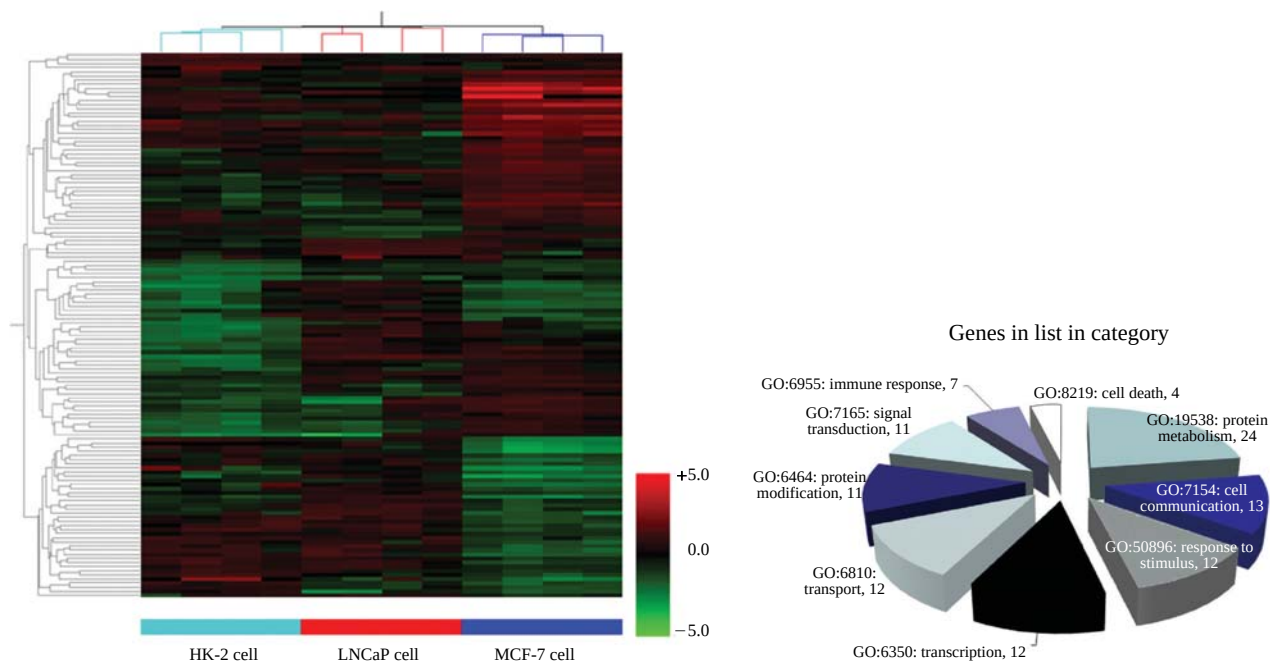


**Figure 3.** Functional classification of differentially expressed genes in NP-treated LNCaP cell (A) low dose-3 hours vs (B) high dose-48 hours.

of high dosage and longer-term experiments, the expression pattern of genes associated with apoptosis, cell growth, and cell proliferation was observed (as is shown in Figure 3). Also, HK-2 cells with low dosage and a short exposure time evidenced changes in the expression of genes related to development-this result was dissimilar to those observed with high dosage and long exposure conditions. However, estrogenic responses resulted in changes in the expression of genes associated with cell proliferation, defense, and metabolism. In the MCF-7 cell line, when the estrogen receptor is activated, expressional changes of genes involved in apoptosis, cell growth, and cell proliferation (Figure 4) are observed as a consequence of high dosage and long exposure time, as compared to the normal cell line.

### The Gene Expression Profiles for the NP in MCF-7 and LNCaP Cell

In MCF-7 cells under low-dose and short-exposure time conditions, the conditions most relevant to the nature of reaction for exposure to small quantities of EDCs, changes in the expression of a group of genes involved in development were observed in large numbers. These genes have been associated with signaling, transcription, and growth in response to estrogen. In the LNCaP cell line, in which the estrogen receptor is not activated, changes in the expression of



**Figure 4.** Comparative hierarchical clustering & functional classification of common regulated genes in HK-2, MCF-7, and LNCaP cells.

genes involved in development were observed under high-dosage and long exposure time, similar to the results of experiments with low-dose and short exposure time samples of the MCF-7 cell line, in which the estrogen receptor is activated. Additionally, genes associated with stress and immune responses evidenced profound expressional changes. In addition, genes associated with cell cycle and cell proliferation also evidenced significant expressional changes. This is believed to occur due to the type of host cell, in which the androgen receptor is activated even when the estrogen receptor is not activated. As a result of an experiment utilizing low-dose and short exposure time conditions in the LNCaP cell line, genes associated with protein modification and biosynthesis evidenced significant expression, and the changes in the expression of genes associated with mitosis and the cell cycle appeared meaningful (Figure 3). The SOP method, as described by Kim *et al.* (2007), was utilized for related pathway analysis<sup>14,15</sup>.

For the analysis of expression changes, we utilized one-way ANOVA (analysis of variance) for statistical analysis, taking time as a parameter analysis, followed by the application of Welch's t-test with a p-value of  $<0.001$ . Via multiple tests of Benjamini and Hochberg False discovery rate, the numerical value of each gene was determined. The results of ANOVA confirmed that a total of 132 genes evidenced expression changes in all cell lines according to time. We also confirmed clear differences according to time, and this was noted in all cell lines in common.

In the biological category, functional classification with an increase and decrease of treatment time in these 3 host cell lines, genes associated with protein metabolism for nonylphenol-related protein and by-products and genes associated with transcription, cell transport, and communication of the estrogen receptor signal cascade were all confirmed. As time increased, expression changes involved in cell death and immune response were also noted in common in these three cell lines. These genes were primarily identified as ribosomal proteins involved in protein biosynthesis, and evidenced differences in their reactions to the nonylphenol contained in each of the cell lines (Data not shown).

## Discussion

In this study, we utilized a human whole genome microarray to identify differentially expressed genes significantly induced by NP in HK, MCF-7, and LNCaP cells (Figure 4). We detected 132 commonly regulated genes on the basis of the results of our 2.0

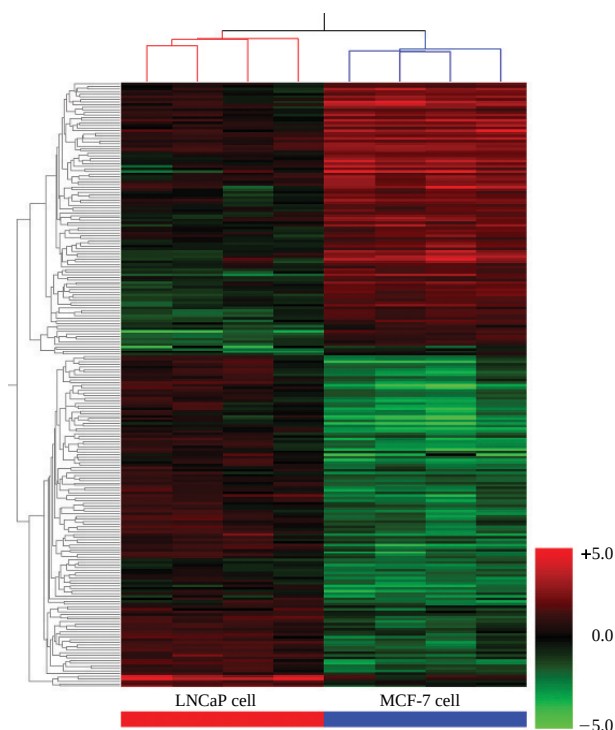
**Table 2.** Functional analysis of estrogen responsiveness-related common genes in human cells (HK, MCF-7, and LNCaP).

| Category                                                                    | Genes in list in category | p-value |
|-----------------------------------------------------------------------------|---------------------------|---------|
| GO:6508: proteolysis<br>CTSH, BLMH, PRSS8, AGA, USP24                       | 14                        | 0.0035  |
| GO:44237: cellular metabolism<br>ASNS, CHST14, NP, IDH1, RPL3, RPS11, HSBP8 | 82                        | 0.015   |
| GO:6936: muscle contraction<br>ADRB2, RYR2, TNNC1, MYOM1, SLMAP             | 5                         | 0.0218  |
| GO:42592: homeostasis<br>BAX, CLCN3, TXNDC2, MYC                            | 7                         | 0.0303  |
| GO:6259: DNA metabolism<br>PEO1, SOX2, XRCC2, JMJD1A                        | 12                        | 0.0333  |
| GO:50896: response to stimulus<br>ATP6V1B1, SP2, THBS1, DUSP10, CYB5R4      | 29                        | 0.0409  |
| GO:9058: biostnthesis<br>NME2, MKNK1, TM7SF2, RRM2B, COQ5                   | 17                        | 0.0928  |
| GO:42981: regulation of apoptosis<br>CD70, DAP3, CASP1                      | 7                         | 0.139   |
| GO:7155: cell adhesion<br>AZGP1,CDH4, MAGI1, LPXN, BYSL                     | 8                         | 0.359   |

fold microarray analysis. In order to verify the relationship between nonylphenol and the estrogen receptor, we conducted a statistical analytical comparison of the MCF-7 cell line, which is an estrogen receptor (+), and the LNCaP cell line, which is an estrogen receptor (−), via 1-way ANOVA. As a result, 235 genes ( $p\text{-value} < 0.00001$ ) evidencing differences in their reactions to nonylphenol according to the activation of estrogen receptor were verified. In these 235 expressed genes, several genes evidencing receptor activity were associated with signal transduction, and some were related to catalase, evidencing RNA binding activity and nucleotide metabolism (Table 2).

As a result of our overall analysis of the entire experiment, the treatment of nonylphenol was shown to induce marked expression changes according to the cell line utilized. Additionally, while the HK-2 normal kidney cells and MCF-7 cells in which the estrogen receptor is activated evidenced similar expression changes, the LNCaP estrogen receptor (−) cells evidenced profound expression during the early stages of the experiment and exhibited some differences from other cell lines. When we evaluated this phenomenon in more detail, both the estrogen receptor 1 and 2 genes evidenced expression only in the MCF-7 cells, and the androgen receptor (AR) gene evidenced





**Figure 5.** Comparative Hierarchical clustering of the gene expression in MCF-7 vs LNCaP cell lines. The color red indicates upregulation, green indicates down-regulation, and black indicates no change.

significant expression in three kinds of samples. The PDZK1 gene, which is generally recognized as an estrogen-responsive gene, evidenced overexpression, just as has been reported in many previous studies (Figure 5). This result was the same as that noted in an experiment of E-screen density targeted toward the detection of small-quantity exposure and in the case of the expression pattern in the MCF-7 cell line with nonylphenol treatment. In this study, many genes involved in cell proliferation, transcription, and signaling were included, and the results were identical to the results of previous experiments (Data not shown).

In order to confirm the utility of this experiment, its mutual meaningfulness with previously published data was verified. As comparison objects, the data of Inoue *et al.*, which had confirmed the nature of the reaction of estrogen material in each cell line was utilized. As the result of comparison analysis, as described in the previous study of Inoue *et al.* in which genes reacting to NP (estrogen receptor antagonist) evidenced different expressional changes in accordance with the cell line tested, the gene group manifesting different reactions in three different cell lines was confirmed<sup>16</sup>. Additionally, these data were

correlated strongly with previously published data<sup>17-20</sup>.

In our comparisons of expression changes with those reported previously by Inoue *et al.*, over 60% were identical per each category as a whole, a result of 77% conformity was obtained. DNA microarrays are a powerful, high-throughput tool for screening the expression of thousands of genes simultaneously. In environmental monitoring, microarrays provide not only a method for the rapid categorization of chemicals and the assignment of a mode of toxic action, but also allow more sensitive end points to be addressed. Recently, a great deal of interest has focused on the use of microarrays in toxicology to quickly classify toxicants based on characteristic expression profiles in a variety of species, and on the use of these profiles as a means for identifying the putative mechanism of action<sup>21-24</sup>. In conclusion, our results may establish a framework for understanding the mechanisms underlying the toxicity of NP.

## Methods

### Chemicals and Reagents

Nonyl phenol (NP) and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich Chemical Company (USA). RPMI-1640 Culture Medium, Dulbecco's Phosphate Buffered Saline (PBS) and Fetal Bovine Serum (FBS) were products of GIBCO™ (USA). All other chemicals used were of analytical-grade or the highest available grade.

### Cell Line and Culture

MCF-7 breast cancer cell were genuine human estrogen-sensitive cells, and remain quiescent when inoculated into ovariectomized hosts. They require the presence of estrogen to grow as a tumor in hosts. When MCF-7 cells are cultured in medium supplemented with nonestrogenic charcoal-stripped human serum, proliferation is prevented. When estrogen is added, the cells proliferate. The LNCaP cells originated from a lymph node metastatic lesion of human prostatic adenocarcinoma, and have been extensively employed in studies of prostate cancer. The androgen-responsive feature of LNCaP also makes it a useful model in studies of the transcriptional regulation of prostate-associated genes, as the expression of many prostate-specific proteins requires functionally differentiated, androgen-responsive cells.

HK-2 Cell (human kidney) is a proximal tubular cell line derived from the normal kidney. Studies evaluating the mechanisms relevant to proximal tubular cell physiology and pathophysiology have in-

creasingly utilized cell culture systems in order to compensate for the complexity of whole organ/whole animal experiments. A primary PTC culture from normal adult human renal cortex was exposed to a recombinant retrovirus containing the HPV 16 E6/E7 genes, thereby resulting in the cell line designated HK-2 (human kidney-2).

The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen Life Technologies) supplemented with 5% fetal bovine serum (Invitrogen Life Technologies), DMEM with 10% fetal bovine serum, and RPMI 1640 medium (Invitrogen Life Technologies) with 10% fetal bovine serum, 100 IU/mL of penicillin, and 100 µg/mL of streptomycin. Subculturing was conducted every 3 or 4 days so as not to exceed  $1 \times 10^6$  cell/mL.

### Determination of Cell viability

An MTT assay was conducted for the detection of cell viability. 24-well plates were utilized for the cytotoxicity assay. MCF-7, HK-2, and LNCaP were seeded at a seeding density of  $2 \times 10^4$  cell/mL.  $5 \times 10^4$  cells/mL and  $5 \times 10^4$  cells/mL cells were exposed to various concentrations of nonylphenol in culture medium at 37°C for 3 h or 48 h of exposure time. 300 µL of MTT (5 mg/mL in PBS) solution was added to each tube and incubated for 3 hrs. DMSO was added to each tube and transferred into 96-well plate. The optimal density (OD) of the purple formazan product was measured at a wavelength of 540 nm. The 20% inhibitory concentration (IC<sub>20</sub>) of cell proliferation in a particular chemical was defined as the concentration required to induce a 20% reduction in cell viability as compared to the solvent-treated controls. The IC<sub>20</sub> values were directly determined from the linear dose-response curves.

### RNA Extract from the Sample

Total RNA was extracted from Nonylphenol-treated human cells (HK-2, MCF7, LNCaP) using a TRI REAGENT (MRC, OH) in accordance with the manufacturer's instructions. Genomic DNA was removed using an RNase-free DNase set (Qiagen, USA) during RNA purification. Each RNA concentration was quantified using Nanodrop, and the quality of the RNA was verified using an Agilent bioanalyzer 2100 (Agilent Technologies, Inc., CA).

### Preparation of Fluorescent DNA Probe and Microarray Hybridization

Gene expression analysis was conducted with the RNA samples using a human whole 44 k microarray (Agilent Technologies, CA, USA).

Each extracted total RNA sample (30 µg) was la-

beled with Cyanine (CY3) or Cyanine (Cy5) conjugated dCTP (Amersham, Piscataway, NJ) via a reverse transcription reaction using reverse transcriptase, SuperScrip II (Invitrogen, Carlsbad, California). The labeled cDNA mixture was then concentrated via an ethanol precipitation technique. The concentrated Cy3 and Cy5 labeled cDNAs were resuspended in 10 µL of hybridization solution (GenoCheck, Korea). After the two labeled cDNAs were mixed, placed on Agilent Human 4 × 44 K whole genome array (G 4112F, Agilent Technologies, Inc., CA) and covered by a MAUI FL chamber (Biomicro systems, Inc. UT). The slides were hybridized for 12 hr at 62°C in the MAUI system (Biomicro systems, Inc. UT). The hybridized slides were washed in 2 × SSC, 0.1% SDS for 2 min, 1 × SSC for 3 min, and 0.2 × SSC for 2 min at room temperature. The slides were then centrifuged at 3,000 rpm for 20 sec to dry.

### Microarray Data Analysis

Hybridized slides were scanned with an Axon Instruments GenePix 4000B scanner (Axon, CA) and the scanned images were analyzed with the GenePix Pro 5.1 (Axon, CA) and GeneSpring GX 7.3.1 (Silicongenetics, CA) programs. Spots judged as substandard by visual examination of each slide were flagged and excluded from further analysis. Spots with dust artifacts or spatial defects were manually flagged and excluded. In order to filter out the unreliable data, spots with signal-to-noise (signal-background-background SD) ratios below 10 were not included in the data. Data were normalized by Global, lowess, print-tip and scaled normalization for data reliability. Fold change filters included the requirement that the genes be present in at least 200% of controls for upregulated genes and lower than 50% of controls for downregulated genes. The data were clustered groups of genes that behaved similarly across the drug treatment experiments, as assessed using GeneSpring GX 7.3.1 program (Silicongenetics, CA). We utilized an algorithm, based on the Pearson correlation, to separate genes of similar patterns. Differentially expressed genes were analyzed via GO functional classification and the KEGG pathway.

### Acknowledgements

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