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# Effects of Dioxin Exposed in Human by Using Radioactive cDNA Microarray

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## Abstract

2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) are well known as the most toxic environmental compound in these days. Many researches are reported that dioxin produces multiple toxic effects, such as endocrine toxicity, reproductive toxicity, immunotoxicity and cancer. In this study, we carried to discover novel evidence for previously unknown gene expression patterns in human exposed to dioxin by using radioactive cDNA microarray. 548 workers who were divided into experimental and control groups according to their urinary Naphthol levels were enrolled in our study. Blood mRNA in human was isolated, and the gene expression profiles were analyzed by cDNA microarray. Gene expression analysis identified 52 genes which exhibited a significant change. In our study, most notably, genes involved in cell cycle, cell proliferation, signal transduction and apoptosis in human exposed to dioxin, such as CCND3, TSHR, and EFRN5, were up-regulated. In the current study, we observed gene expression of people that are exposed to dioxin using radioactive cDNA microarray. Through these results, we suggest when objects are exposed to toxic compounds, such as dioxin, the radioactive cDNA microarray may be using in sensitively detecting of cancerous change.

**Keywords:** Dioxin, Radioactive cDNA microarray, Gene expression

2, 3, 7, 8-Tetrachlorodibenzo-*p*-dioxin (TCDD) are well known as the most toxic environmental compound in these days. These environmental pollutants include polycyclic aromatic hydrocarbons (e.g., benzo[*a*]pyrene), polyhalogenated hydrocarbons, dibenzofurans, and the most potent small-molecular-weight toxicant known, dioxin[1,2]. Many researches are reported that dioxin produces multiple toxic effects, such as endocrine toxicity, reproductive toxicity, immunotoxicity and cancer[5]. TCDD and related compounds are legacy environmental contaminants that cause controversial human health effects at environmental levels[3].

Toxicogenomic studies have shown that both large-scale measurement of gene expression (transcriptomics) and metabolite levels (metabolomics) complement the current methods to identify and discriminate different types of toxicity[4]. cDNA-microarray has become an important tool in toxicogenomics. High-throughput measurement of transcriptional changes that occur as a consequence of xenobiotic exposure is facilitating the elucidation of toxicological mechanisms[6]. This technology is used for identifying the gene expression regulated by mutagenic, carcinogenic and toxic xenobiotics such as TCDD, which causes various biological and toxic responses[7]. The main advantage of this approach is the uniformity of the end results and hence the comparability of the data among different gene expression products[8]. Basically, the mRNA expressions are the same criteria used by most of cDNA microarray approaches today, and therefore we judge that this is the most straight forward and simplest approach which allows us to compare many gene products for each time point all at once.

### General Characteristics and Urinary Naphthol Levels of Subjects

Table 1 shows general characteristics of subjects such as age. There are no significant differences of age distribution between control and experimental groups.

As shown in Table 2, urinary Naphthol levels were significantly higher in experimental group ($9.21 \pm 1.93$ μmol/mol creatinine) than those in control group ($1.95 \pm 1.66$ μmol/mol creatinine).

### Superimposed Image Analysis in Dioxin-exposed Human Subject

Radioactive hybridization was visualized by phos-

**Table 1.** Characteristics of control and experimental groups.

| | Control groups (N=521) | Experiment groups (N=27) | Significance |
|---|---|---|---|
| Age (year old) | | | |
| 20-29 | 176 (33.8%) | 7 (25.9%) | $\chi^2$=1.005 |
| 30-39 | 174 (33.4%) | 11 (40.7%) | P=0.800 |
| 40-49 | 123 (23.6%) | 6 (22.2%) | df=3 |
| 50< | 48 (9.2%) | 3 (11.1%) | |

**Table 2.** Levels of 2-naphthol in control and experimental groups.

| | Control groups (N=521) | Experiment groups (N=27) | P-value |
|---|---|---|---|
| 2-Naphthol (μ mol/ mol creatinine) | 1.95±1.66 | 9.21±1.93 | 0.000 |

phoimager technologies. The primary image, which is the result of primary capture by the phosphoimager, is shown in Figure 1A. This particular array was printed in duplicate and each duplicate was composed of eight individual subarrays. Visual inspection of the hybridization patterns readily identified a number of signals differentially expressed between experimental and control group. Fig. 1B is a superimposed image in which the red color represents up-regulation, the green represents down-regulation in experimental group and the yellow represents genes of higher expression in experimental group and the control group, such as housekeeping genes. Analysis of the median densitometric signal intensity revealed that 52 genes differed between experimental and control group by a Z-ratio of 2 at the descriptive $P \leq 0.05$. Two genes were marked: two genes differentially expressed between experimental and control group (red, G1/S-specific cyclin D3; green, tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain).

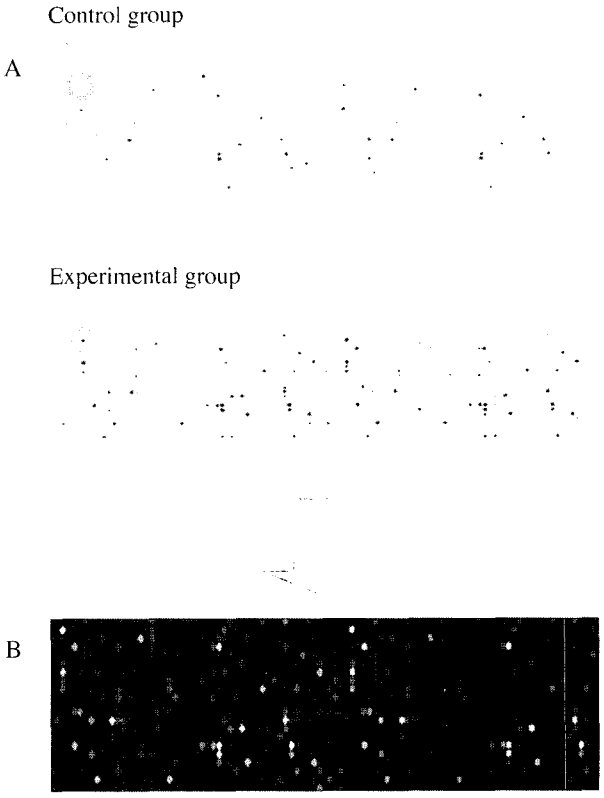


**Fig. 1.** Superimposed image. A, Representative cDNA microarrays of two independent hybridization experiments comparing cDNAs generated from controls group (up) or from experimental group (down). The cDNA microarray contained the two sets of 1,152 genes and printed in duplicate, and each duplicate is composed of eight individual subarrays. For example, two genes differentially expressed between control and experimental groups are marked by circles (red, G1/S-specific cyclin D3; green, tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain). B, Superimposed image of primary images of control and experimental groups.

## Gene Expression Profiles in Dioxin-exposed Human Subject

Using the cDNA expression array, we found that among 1,152 genes on the array membrane, 20% of total genes presented a quantifiable expression in experimental group. Gene expression profiles of interest were significantly up-regulated or down-regulated in experimental group when compared with control group. Genes showing highly altered expression levels were aligned in the order of magnitude of altered expression in experimental group. The up- and down-regulated genes are listed in Table 3. Gene expression profiles showed that 16 genes were up-regulated in experimental group; e.g., chemokine (C-C motif) receptor 5, Bruton agammaglobulinemia tyrosine kinase, G1/S-specific cyclin D3. Gene expression profiles showed that 36 genes were down-regulated in experimental group; e.g, Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain (TNFRSF10D), Bcl-2-related protein A1 (BCL2A1) and Caspase-3 (CASP3).

Figure 4 is a Scatter plot for comparing the expression profiles between experimental and control group. Expression profiles of experimental and control group are shown as a scatter plot of 1,152 genes from the microarray. Regression analysis of Z scores from two independent samples of experimental and control group was performed and Z scores of indivi-

**Table 3.** Up- and Down-regulated gene expression in experimental group.

| Up-Gene name | Gene symbol | Z-ratio |
|---|---|---|
| Chemokine (C-C motif) receptor 5 | CCR5 | 4.14 |
| Bruton agammaglobulinemia tyrosine kinase | BTK | 3.58 |
| G1/S-specific cyclin D3 | CCND3 | 3.19 |
| Mitochondrialcreatinekinase gene | CKMT | 2.96 |
| Thyroid stimulating hormone receptor | TSHR | 2.96 |
| Proteinkinase | ZPK | 2.86 |
| Src kinase-associated phosphoprotein of 55 kDa | SCAP1 | 2.71 |
| Mitogen-activated protein kinase 11 | MAPK11 | 2.53 |
| Ephrin-A5 | EFRN5 | 2.32 |
| Phosphatidylinositol-4-phosphate5-kinasetypeIIbeta | PIP5K2B | 2.21 |
| NF-E2-related facotr | NF-E2 | 2.17 |
| Proteinphosphatase2 (formerly2A), regulatorysubunitB, alphaisoform | PPP2R2C | 2.15 |
| Calmodulin 3 (phosphorylase kinase, delta) | CALM3 | 2.12 |
| Lectin,galactoside-binding, soluble, 7 (galectin7) | LGALS7 | 2.10 |
| LIMdomainkinase1 | LIM | 2.09 |
| Transcriptionfactor11 (basicleucinezippertype) | BATF | 2.07 |
| *Down-Gene name* | Gene symbol | Z-ratio |
| dual-specificity tyrosine (Y) phosphorylation regulated kinase 4 | DYRK4 | −8.44 |
| "Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain" | TNFRSF10D | −7.00 |
| Proteinphosphatase6 | - | −6.37 |
| Bcl-2-related protein A1 | BLL2A1 | −4.75 |
| Ndrproteinkinase | AIP | −4.66 |
| ImmunophilinhomologARA9 | - | −4.65 |
| "Major histocompatibility complex, class I, A" | HLA-A | −4.58 |
| ESTs,ModeratelysimilartoSERINE/THREONINE-PROTEINKINASEPAK | - | −4.46 |
| "Ras homolog gene family, member D" | RHOD | −4.46 |
| TranscriptionfactorAP-4(activatingenhancer-bindingprotein4) | TFAP4 | −4.40 |
| "Burkitt lymphoma receptor 1, GTP-binding protein; CXCR5" | BLR1 | −4.33 |
| (CloneN5-4)proteinp84 | - | −4.08 |
| L-3-phosphoserine-phosphatasehomologue | PSPHL | −4.03 |
| Interferon regulatory factor 6 | IRF6 | −4.01 |
| Lumican | LUM | −3.96 |
| "MAD (mothers against decapentaplegic, Drosophila) homolog 6; Smad6" | SMAD6 | −3.86 |
| Colony stimulating factor 3 receptor (granulocyte) | CSF2RB | −3.80 |
| "Major histocompatibility complex, class II, DM beta 1" | HLA-DMB | −3.65 |
| Platelet-derivedgrowthfactorreceptor,betapolypeptide | PDGFB | −3.56 |
| CLEAVAGESIGNAL-1PROTEIN | - | −3.50 |
| Ovalbumin Transcription Factor I (nuclear receptor subfamily 2, group F, member 1) | NR2F1 | −3.50 |
| NKG2-DTYPEIIINTEGRALMEMBRANEPROTEIN | LOC611355 | −3.28 |
| CCAAT/enhancer-binding protein epsilon (C/EBP epsilon) | CEBPE | −3.24 |
| CDC28proteinkinase2 | CKS2 | −3.16 |
| PhosphodiesteraseI/nucleotidepyrophosphatasebeta | PDNP3 | −3.13 |
| DNA (cytosine-5-) methyltransferase 3 beta | DNMT3B | −2.94 |
| Protein tyrosine kinase 2 | PTK2 | −2.82 |
| "Glutamate receptor, ionotropic, N-methyl D-aspartate 2C" | GR1N2C | −2.63 |
| Cytochrome P450 IIF1 | CYP2F1 | −2.47 |
| ESTs, ModeratelysimilartoPROTEIN-TYROSINEPHOSPHATASE | - | −2.42 |
| "Caspase 3, apoptosis-related cysteine protease" | CASP3 | −2.23 |
| ESTs, HighlysimilartoCALCIUM/CALMODULIN-DEPENDENTPROTEINKINASEGR | - | −2.21 |
| Insulin-like growth factor 2 receptor | IGF2R | −2.20 |
| Delta-like protein precursor | DLK | −2.18 |
| RAP1A,memberofRASoncogenefamily | RAP1A | −2.10 |
| Proline-serine-threonine phosphatase interacting protein 1 | PSTPIP1 | −2.05 |

[1]$Z\text{-ratio}_{(gene1)} = Z\text{-difference}_{(gene1)} / Sdev_{(Z\text{-difference all genes})}$; Fold of expression change for an individual gene based on the ratio for the experimental group compared to that of the control group.

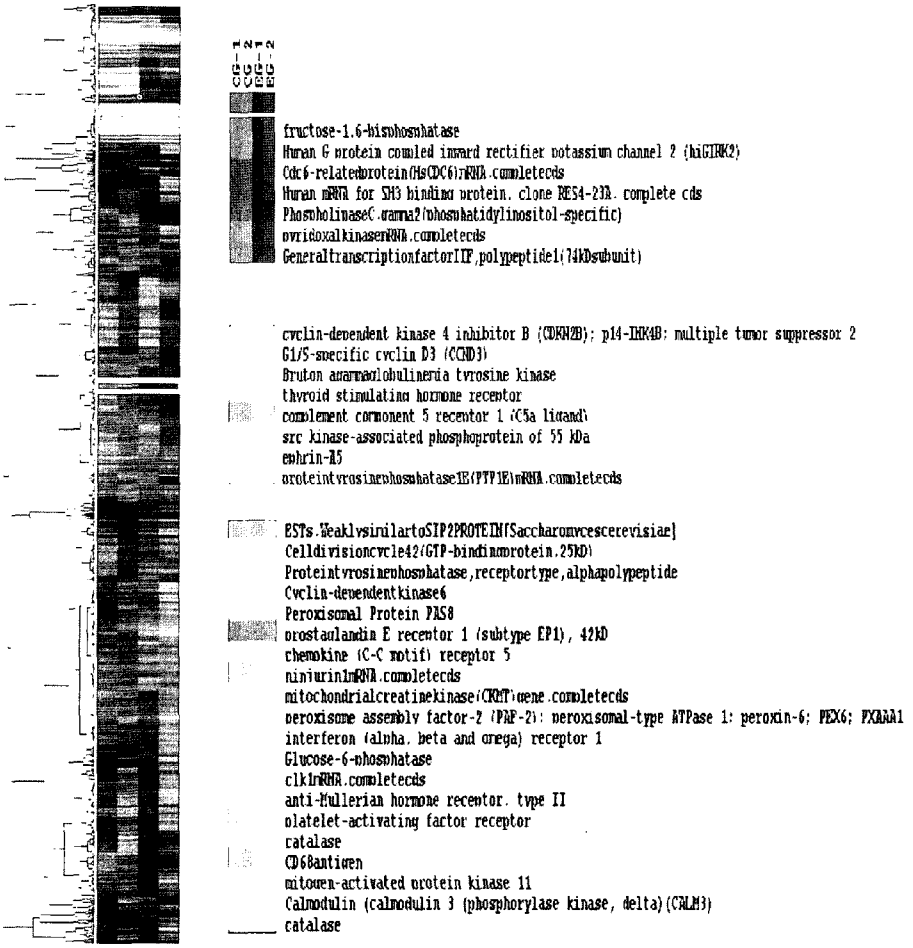


**Fig. 2.** Clustergram of up-regulated genes in experimental groups. Microarray data from control group and experimental group exposed to dioxin were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate programs available as shareware from Michael Eisen's lab. Each gene is represented by a single row of colored boxes; each experimental sample is represented by a single column. The entire clustered image is shown on the left. These clusters contain uncharacterized genes and genes not involved in these processes.

dual genes were plotted.

To obtain a molecular portrait of the relationships between the metabolism associated with experimental group, we used a hierarchical clustering algorithm to group genes on the basis of similar expression patterns and the data is presented in a matrix format (Figs. 2, 3). Each row of Figure 2 and Figure 3 represents the hybridization results for a single DNA element of the array and each column represents the expression levels for all genes in a single hybridization sample. The expression level of each gene was visualized in color, relative to its median expression level across all samples. Red represented expression greater than the mean, green represents expression less than the mean and color intensity denotes the degree of deviation from the mean. Gray represented the median expression level. Distinct samples representing similar gene patterns from control cells were aligned in adjacent rows. The cells included in this map were samples from right experimental and control group. Coordinately expressed genes were grouped into clusters, which were named on the basis of the cellular process in which the component genes participated in. The clustergram revealed that clusters of genes related to progression were up- and down-regulated in experimental group, as compared to control group (Figs. 2, 3).

## Discussion

The International Agency for Cancer Research on Cancer (IARC) have classified dioxins as a human carcinogen[4]. TCDD is a prototype and the most potent chemical of the polychlorinated dibenzo-*p*-dioxins and polychlorinated dibenzofurans (dioxins).

The purpose of this study is to evaluate profiles of gene expression in dioxin exposed human subjects by using the results of cDNA microarray analysis.

In our epidemiological study, most notably, genes involved in cell cycle such as CCND3 and EFRN5, cell proliferation such as CCR5, TSHR and genes involved in signal transduction related several genes in subjects with higher serum naphthol levels were

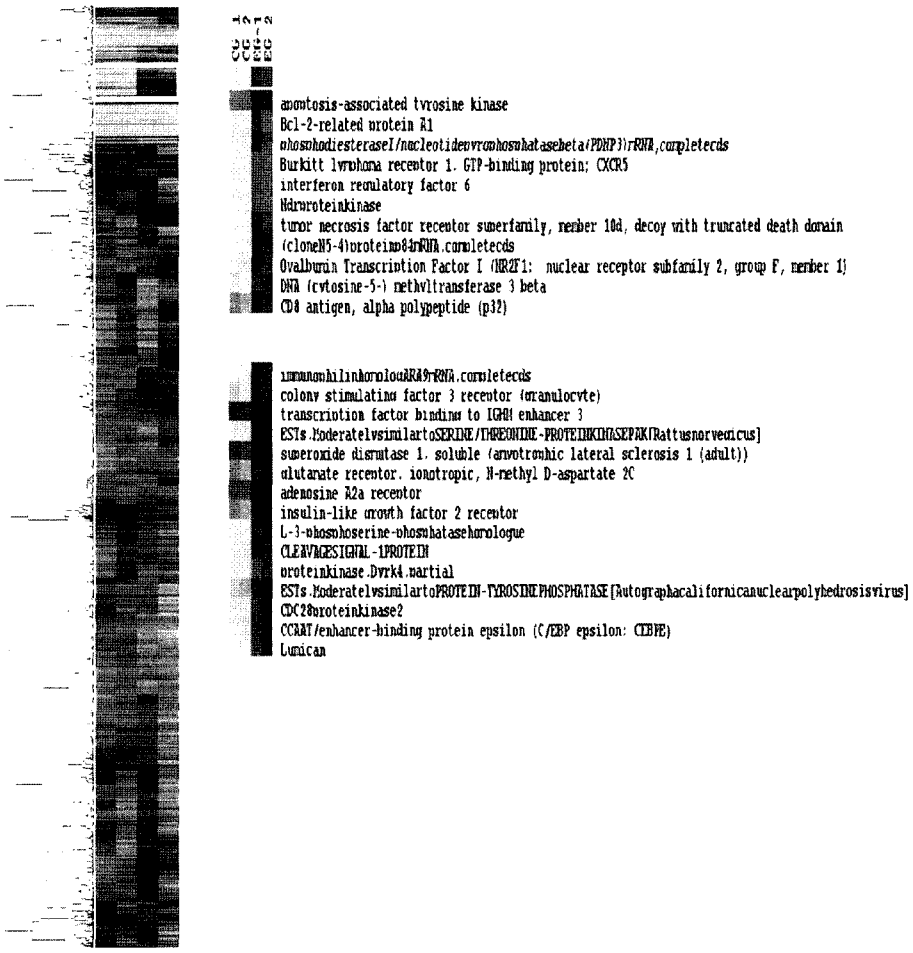


**Fig. 3.** Clustergram of down-regulated genes in experimental groups. Microarray data from control group and experimental group exposed to dioxin were combined and clustered. Cluster analysis was performed on Z-transformed microarray data using two separate programs available as shareware from Michael Eisen's lab. Each gene is represented by a single row of colored boxes; each experimental sample is represented by a single column. The entire clustered image is shown on the left. These clusters contain uncharacterized genes and genes not involved in these processes.

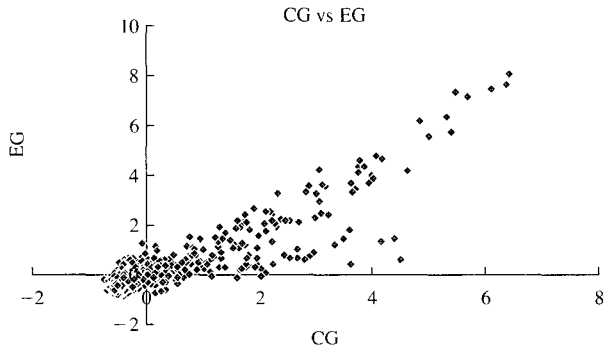


**Fig. 4.** Scatter plot for comparison of expression profile between control and experimental group. Expression profiles of control and experimental groups are shown as bivariated scatter plot of 1,152 genes from the microarray. The values are corrected intensities relative to control, representing levels of expression for the cDNA elements of the microarrays.

up-regulated in dioxin-exposed human subjects. Other previous researchers have reported that these up-regulated genes were related with development or metastasis of various cancer[14].

In our study, the G1/S-specific cyclin D3 (CCND3) and Ephrin-A5 (EFNA5), whose members are characterized by a dramatic periodicity in protein abundance through the cell cycle, was up-regulated.

CCND3 plays a pivotal role in tightly controlling the physiologic progression from the $G_1$ to the S phase of the cell cycle[14]. After assembling with cyclin-dependent kinases 4 and 6, cyclin D3 is capable of phosphorylating the retinoblastoma gene product, eventually promoting the entry of the cell into the S phase. Cyclins function as regulators of CDK kinases. Different cyclins exhibit distinct expression and degradation patterns which contribute to the temporal coordination of each mitotic event. This cyclin forms a complex with and functions as a regulatory subunit of CDK4 or CDK6, whose activtiy is required for cell cycle G1/S transition. This protein has been shown to interact with and be involved in the phosphorylation of tumor suppressor protein Rb. Especially, Cyclin D3 has a potential in cancer since it is overexpressed

in different human cancers and rearrangements of cyclin D3 gene have been observed in malignancies of the lymphoid system[15]. Similarly to *cyclin D1*, *cyclin D3* is likely to be a specific target of genetic abnormalities, such as the t(6;14)(p21.1;q32.3) translocation in a fraction of plasma cell myelomas and non-Hodgkin's lymphomas and gains of the 6p21 locus in several different malignancies, including ovarian and gastric adenocarcinoma[14]. It has been shown that *cyclin D3* overexpression renders cells prone to malignant transformation[14,16].

EFRN5, a member of the ephrin gene family, prevents axon bundling in cocultures of cortical neurons with astrocytes, a model of late stage nervous system development and differentiation. Also, Iida *et al.*[17] demonstrated that ephrin-A1 induced expression of genes related to the cell cycle, angiogenesis (angiopoietin 1 and thrombospondin 1), and cell-cell interactions (Rho, integrin, and matrix metalloproteinases) in cultured hepatoma cells. It has been reported that eph receptor protein tyrosine kinases and their ligands, ephrins, are associated with increased tumorigenicity in patients with breast carcinoma and melanoma[18]. It is reasonable to expect the TCDD-induced tumorigenecity to involve alterations in cell signaling[4].

Cell Proliferation genes such as chemokine (C-C motif) receptor 5 (CCR5) and thyroid stimulating hormone receptor (TSHR) were up-regulated in this study.

TSHR were related to cell proliferation, cell differentiation, and apoptosis[19]. TSHR expression is frequently silenced in epithelial thyroid cancers associated with decreased or absent TSH-promoted iodine uptake[20].

CCR5, a member of the beta chemokine receptor family, are implicated in tumor pathogenesis, although it is unclear whether they affect human cancer progression positively or negatively[21]. We found that activation of the chemokine receptor CCR5 regulates p53 transcriptional activity in breast cancer cells through pertussis toxin-, JAK2-, and p38 mitogen-activated protein kinase-dependent mechanisms[22]. Many cancers express an extensive network of chemokines and chemokine receptors[22,23]. Studies in animal models suggest that chemokines may act on tumor cells and/or tumor-infiltrating leukocytes[36]. Tumor-produced chemokines are thought to have distinct roles in the biology of primary and metastatic disease, including directing leukocyte infiltration into the tumor, regulating the antitumor immune response, controlling tumor angiogenesis, functioning as autocrine or paracrine growth and survival factors, and controlling tumor cell movement[21]. Current evidence does not establish whether these chemokine biological activities in the tumor microenvironment contribute to cancer growth and spread, or to host antitumor response and cancer regression. Studies using the CC chemokine CCL5 (RANTES) as a model reported that elevated CCL5 levels in the tumor environment contribute to improving the immune response against breast carcinomas, but also correlate with poor prognosis in breast cancers[25]. CCL5 also has a role in the chemotaxis and metastasis of breast cancer cell lines[26].

The seven genes were differentially expressed in the signal transduction are up-regulated in dioxin-exposed human subjects such as Bruton agammaglobulinemia tyrosine kinase (BTK), mitochondrialcreatinekinase gene (CKMT), mitogen-activated protein kinase 11 (MAPK11), phosphatidylinositol-4-phosphate 5-kinasetype II beta (PIP5K2B), Protein-phosphatase2, regulatorysubunitB, alphaisoform (PPP2R2C) and Calmodulin (calmodulin 3 (phosphorylase kinase, delta) (CALM3).

Btk is a cytoplasmic protein tyrosine kinase (PTK), belonging to the Tec family[27] and plays an important role in B-cell development and signaling[28,29]. A form of X-linked immunodeficiency is caused by the mutation of Btk both in human and mouse. The cytoplasmic PTKs consist of several families, the largest being the Src and the Btk/Tec families. A wide body of data demonstrate the involvement of PTKs in important cellular signaling processes in T and B cells[30]. Genetic and biochemical analyses indicate that Btk is a component of the signal transduction pathways utilized by the B cell antigen receptor (BCR), IL-5R, IL-6R, IL-10R, CD38, and FceRI. Activation of Btk by either receptor crosslinking or a point mutation E41K in the PH domain (Btk) is dependent on Src family kinases and is correlated with increased tyrosine phosphorylation[29]. Reactive oxygen species (ROS) or reactive oxygen intermediates (ROIs) mediate signaling in many different cell types. During aerobic energy metabolism in the mitochondrial electron transport, $O_2$ is reduced to $H_2O$. Partially reduced and highly reactive metabolites of $O_2$, ROS may be formed during these electron transfer reactions[31]. These include superoxide anion ($O_2^-$) and hydrogen peroxide ($H_2O_2$), which are converted to $H_2O$ by antioxidant enzymes such as superoxide dismutase and glutathione peroxidase. Although oxidative stress has been known to be involved in cancer, neurodegenerative diseases, and aging, recent studies have shown that ROS also serve as important signaling molecules and function as second messengers[32].

CKMT is responsible for the transfer of high energy phosphate from mitochondria to the cytosolic car-

rier, creatine. It belongs to the creatine kinase isoenzyme family. It exists as two isoenzymes, sarcomeric MtCK and ubiquitous MtCK, encoded by separate genes. Mitochondrial creatine kinase occurs in two different oligomeric forms: dimers and octamers, in contrast to the exclusively dimeric cytosolic creatine kinase isoenzymes. Under situations of compromised cellular energy state, which are often linked to ischemia, oxidative stress and calcium overload, two characteristics of mitochondrial creatine kinase are particularly relevant: its exquisite susceptibility to oxidative modifications and the compensatory up-regulation of its gene expression, in some cases leading to accumulation of crystalline MtCK inclusion bodies in mitochondria that are the clinical hallmarks for mitochondrial cytopathies.

Many malignant cancers with poor prognosis have shown overexpression of ubiquitous mitochondrial creatine kinase; this may be related to high energy turnover and failure to eliminate cancer cells via apoptosis. Tokarska-Schlattner *et al.* suggest that CKMT, a key enzyme in cellular energy metabolism, could be involved in anthracycline cardiotoxicity[33].

The MAPK 11 signal transduction pathway is an important, well-studied pathway in cell signaling which controls cell growth. MAPK 11 is a member of the MAP kinase family. MAP kinases act as an integration point for multiple biochemical signals, and are involved in a wide variety of cellular processes such as proliferation, differentiation, transcription regulation and development. This kinase is most closely related to p38 MAP kinase, both of which can be activated by proinflammatory cytokines and environmental stress. Lim S *et al.* suggest a novel cell cycle-dependent function for p38, suppression of the function of Mirk as a transcriptional activator only when cells are proliferating, and thus limiting Mirk function to growth-arrested cells[34]. MAPK cascades transmit and amplify signals involved in cell proliferation as well as cell death, and ERK is most relevant to breast cancer[4]. The Raf-mitogen-activated protein kinase (MAPK or ERK) signaling pathway is well known for its potent capacity to inhibit apoptosis[35]. And Naderi *et al.*[36] suggest that the p38MAPK activation-Bax expression pathway might be involved in apoptosis induced by oxidative stress. Accordingly, the MAPK/ERK pathway, which is well known to promote survival in a number of situations, can protect against apoptosis by inhibiting the overall activation of caspases[37].

PIP5K2B, a member of the phosphatidylinositol-4-phosphate 5-kinase family, encoded protein sequence does not show similarity to other kinases, but the protein does exhibit kinase activity. Additionally, the encoded protein interacts with p55 TNF receptor. Tumor necrosis factor-α (TNF-α) binding to its receptors leads to a diversity of biological responses. Two transcript variants encoding different isoforms have been found for this gene. The protein encoded by this gene catalyzes the phosphorylation of phosphatidylinositol-4-phosphate on the fifth hydroxyl of the myo-inositol ring to form phosphatidylinositol-4, 5-bisphosphate. PIP5K is likely to play a central role in phosphoinositide signal transduction for a variety of growth conditions. The activity of PIP5K versus other signaling activities may determine whether cells respond to TNF by cell proliferation or apoptosis[38].

PPP2R2C is one kind of serine/threonine protein phosphatase regulating mainly cell growth and division. Many cell biological processes are regulated by reversible protein phosphorylation, which is controlled by protein kinases and phosphatases. Among the serine/threonine phosphatases, protein phosphatase 2A (PP2A) plays an important role in the regulation of cell growth and division. Several protein kinases in cellular signal transduction cascades are substrates for PP2A, including protein kinase B, protein kinase C, p70 S6 kinase, calmodulin-dependent kinases, and cyclin-dependent kinase[39].

Also, genes involved in signal transduction related up-regulated genes encoding transcription factor were src kinase-associated phosphoprotein of 55 kDa (SCAP1), NF-E2-related factor (NF-E2), LIMdomainkinase1 (LIM) and Transcriptionfactor11 (BATF).

SCAP1, which protein encoded by this gene belongs to the src family kinases, is a cytoplasmic protein which is preferentially expressed in T-lymphocytes where it interacts with the protein-tyrosine kinase p59fyn. SCAP1 is a member of the Src-family of tyrosine kinases, function of which is closely coupled to growth factor signaling such as PDGFR and EGFR, cell dhesion and cytoskeletal organization[40], cancer etiology[41], as well as hormone signaling. Thus it is possible that such an action of TCDD to activate c-Src kinase activities could cause significant effects on many cellular functions[8].

NF-E2 has been suggested to function as a cofactor for induction of Nqo1 following TCDD exposure, and the results here further demonstrate that NF-E2 is an early and sensitive target for TCDD. Recently a number of studies have implicated the NF-E2 proteins along with the small Maf proteins in binding and modulating the activity of genes carrying antioxidant regulatory elements (ARE)[42]. The consensus ARE core sequence (TGANNNCG), which shows remarkable similarity to the binding sequence of the erythroid NF-E2 binding site, is essential for mediat-

ing the expression of a number of genes encoding enzymes involved in the phase II metabolism of xenobiotics[43]. So far ARE elements have been described in the rat and mouse glutathione S-transferase genes (GST)[44], the rat and human NAD (P)H quinone oxidoreductase[45], the ferritin-L gene, the human heme oxygenase-1 gene[46] and glutamylcysteine synthetase (GCS)[42].

The LIM-domain-binding protein Ldb1 is a key factor in the assembly of transcriptional complexes involving LIM-homeodomain proteins and other transcription factors that regulate animal development. LIM-homeodomain proteins, encoded by *Lhx* genes, are important transcriptional regulators of invertebrate and vertebrate embryonic development[47].

Another more up-regulated gene, Lectin, galactoside-binding, soluble, 7 (galectin 7, LGALS7) is an adhesive protein that beta-galactoside-binding proteins implicated in modulating cell-cell and cell-matrix interactions. Galectins and their ligands have been implicated in cell transformation and cancer metastasis, and found to have prognostic value[48]. Ueda S *et al.*[49] show that galectin-7 has a suppressive effect on tumor growth, suggesting that galectin-7 gene transfer or other means of specifically inducing galectin-7 expression may be a new approach for management of cancers.

Whereas, we found that the genes related with apoptosis such as Tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain (TNFRSF10D), Caspase-3 (CASP3) and Bcl-2-related protein A1 (BCL2A1) were down-regulated in subject with higher serum naphthol levels were down-regulated genes in dioxin-exposed human subjects.

Apoptosis is characteristically executed by cysteine proteases, termed caspases[24]. The most specific way to activate the caspases machinery is through death receptors (DRs), such as the tumor necrosis factor (TNFR), Fas receptor (FasR), and TRAIL (TRAIL-R). The apoptotic signaling is tightly regulated by the balance of pro-apoptotic and anti-apoptotic proteins and an imbalance between cell death and proliferation may cause numerous diseases, including cancers[37].

TNFRSF10D protein encoded by this gene is a member of the TNF-receptor superfamily. This receptor contains an extracellular TRAIL-binding domain, a transmembrane domain, and a truncated cytoplamic death domain. This receptor does not induce apoptosis, and has been shown to play an inhibitory role in TRAIL-induced cell apoptosis.

Caspases-3 is the main executioner of apoptosis responsible for cleavage sites to generate the active enzyme[50]. Despite the central role of caspases in apoptosis, screening for mutations revealed that caspase mutations are a rare event in human tumors. Nevertheless, the expression of caspase-8 can be downregulated by gene deletion or hypermethylation like in some tumors derived from neuroblastomas, malignant brain tumors, or lung carcinomas[51]. In parallel, decreased level of caspases-3 and -8 has been frequently observed in lymphomas. The restoration of caspase-8 or -3 expression can sensitize resistant tumor cells to DR- and drug-induced apoptosis. In addition to the overexpression of anti-apoptotic genes, tumors can develop resistance to apoptosis by downregulation or mutation of pro-apoptotic genes[37].

BCL2A1 is a member of the BCL-2 protein family. The proteins of this family form hetero- or homodimers and act as pro- and anti-apoptotic regulators that are involved in a wide variety of cellular activities such as embryonic development, homeostasis and tumorigenesis. In some cancers including hematopoietic malignancies and colon carcinomas, the mutation of the pro-apoptotic Bcl-2 family member, Bax, is common, leading to a loss of expression or function[37]. The protein encoded by this gene is able to reduce the release of pro-apoptotic cytochrome c from mitochondria and block caspase activation. Bcl-2 family molecules, such as Bid, Bak, and Bax, are involved in the parthenolide-induced apoptosis and that the defective expression of Bcl-X (L) might contribute to the higher parthenolide sensitivity in the SCK cells than in the other adenomatous cholangiocarcinoma cells[52]. If the level of Bcl-2 protein can be reduced sufficiently in tumors using RNA interference (RNAi) to target the gene message, the apoptosis of tumor cells may be promoted[53]. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be selective in the induction of apoptosis in cancer cells with minimal toxicity to normal tissues. However, not all cancers are sensitive to TRAIL-mediated apoptosis. Thus, TRAIL-resistant cancer cells must be sensitized first to become responsive to TRAIL[54].

Therefore, the inhibition of apoptosis by growth factor signaling pathway is one possible mechanism of tumor promotion/progression[4]. Our results which the expression of genes related with cell cycling, proliferation, signal transduction, apoptosis was higher in experimental group than that in control group confirmed above mentioned previous reports, and means that exposure to dioxin attenuated the risk of tumor.

Also, we observed that the expression of genes related with metabolism of dioxin was regulated in experimental groups compared with control group. These were known as dioxin-induced tumorigenic

genes. Kiukkonen *et al.*[1] suggested that the aryl hydrocarbon receptor (AhR) play a central role in the inhibition of apoptosis and the regulation of cell cycle by dioxin. Not only inhibition of apoptosis but also other toxic mechanisms by dioxin were believed to be mediated by alterations in gene expression via activation of the aryl hydrocarbon receptor (AhR). AhR is a transcription factor possessing a subunit for high-affinity binding of TCDD and related compounds[1]. Exposure to dioxin leads to abundant nuclear translocation of AhR, heterodimerization with ARNT (AhR -nuclear translocator), and activation of many genes, including several that encode xenobiotic /carcinogen metabolizing enzymes. Participation of AhR in liver development, nephrogenesis, function of the immune system, cell proliferation, and differentiation as well as retinoic acid metabolism suggests that the AhR may be translocated to the nucleus to regulate those processes also in the absence of exogenous ligands[2].

Many of molecular biology studies show that the aryl hydrocarbon receptor (AhR) acts as a nuclear ligand-induced transcription factor that interacts with xenobiotics such as TCDD. Therefore, TCDD has the potential to directly alter the expression of a large number of genes[3]. The AhR gene also displays affinity for structurally related xenobiotics, including polychlorinated dibenzo-*p*-dioxin (PCDDs), polychlorinated dibenzofurans (PCDFs), and coplanar polychlorinated bipenylas (PBCs)[55]. The initial step in the mechanism of TCDD-toxicity involves binding to the AhR followed by a subsequent increase or decrease in the transcription of AhR-regulated genes[3,56]. Dioxin-AhR complex triggers the dissociation of interacting proteins and results in the subsequent translocation of the ligand-bound AhR to the nucleus, where it heterodimerizes with the aryl hydrocarbon receptor nuclear translocator (ARNT)[57,58]. Thus, the formed AhR/Arnt heterodimer recognizes an enhancer DNA element designated xenobiotic responsive element (XRE) sequence located in promoter regions of target genes, which causes sustained inhibition of intercellular communication in mouse hepatoma cells and induces extensive oxidative damage in cultured cells, in mice, and in female rats.

Activation of the aryl hydrocarbon receptor (AhR) by environmental contaminants such as dioxin is also associated with dioxin-expressed cancerous histologic changes as well as. adverse biological outcomes including regulation of genes related with malignancies.

We also observed that gene expression of Aryl hydrocarbon receptor (AhR), Cytochrome P450 family (CYP450), Glutathione-S-transferase (GST), Diaphorase, carnitine palmitoyl transferase (CPT) and glucose-6-phosphate dehydrogenase (G6PD) were up-regulated by dioxin exposure. Fisher *et al.*[3] reported a similar results previously, which shown to induce the expression of several genes through the TCDD -exposure mechanism, including cytochrome P4501A1 /2, cytochrome P4501B1, glutathione S-transferase Ya, aldehyde dehydrogenase 3 and UDP-glucuronosyl transferase 1, which were called "AhR gene battery"[57].

In conclusion, there is ample evidence showing that the dioxin-induced environmental toxicity and cancer is AhR activation dependent. We demonstrated that dioxins are environmental toxicants of concern due to potent toxic effects on humans, inducing multiple organ pathology and carcinogenesis. Also, we observed that these cancerous regulations of the gene expression in human subjects. Consequently, the expression profiles associated with dioxin could be useful in monitoring human populations in the environment for potential point sources of environmental contamination. The microarray-based genomic survey is a high-throughput approach that allows parallel study on expression patterns of numerous of genes[59]. We concluded that there has been interest in using array in toxicology to quickly classify toxicants based on characteristic expression profiles and to use these profiles as means of identifying putative mechanisms of action.

## Methods

### Subjects

In clinical study, 548 workers who had undergone an annual health examination at Korea University Hospital (Seoul, South Korea) from June 1 to December 31, 2004 were enrolled in our study. All subjects completed a questionnaire, which include items on smoking, age, mediation, etc, and their blood and urine were collected. They had not eaten any grilled and smoked meats within the last 48 h. We divided to control (Urinary Naphthol under 95$^{th}$ confidence upper limit) and Experimental groups (Urinary Naphthol above 95$^{th}$ confidence upper limit) according to their urinary Naphthol concentrations.

### Determination of 2-Naphthol in Urine

Urinary 2-naphthol was analyzed according to the method of Kim *et al.*[9]. Three milliliter of urine samples were buffered with 300 μL of 0.2 M sodium acetate buffer (pH 5.0), and then hydrolyzed enzymatically with 30 μL of β-glucuronidase and sulfatase (Sigma Co. St. Louis, MO, USA), for 16 h at 37°C in

a shaking water bath. After hydrolysis, 5 mL of acetonitrile was added and centrifuged at 1,000 g for 10 min. A 20 μL of the supernatant was injected into the HPLC system. A standard stock solution of 2-naphthol was prepared by dissolving 2-naphthol (Sigma Co. St. Louis, MO, USA) in acetonitrile (Merck Co. Damstadt, Germany). The urinary creatinine was determined with a Hitachi 747 Computer Directed Analyzer.

## Blood Sample Preparation

Blood samples, 4-5 mL of heparinized whole blood, were collected by veinpuncture from each human subject. The human blood plasma and the buffy coat containing white blood cells were isolated from the remaining whole blood by centrifugation (1,000 g, 5 min) and then this was aliquoted and frozen at −70°C until RNA extraction.

## RNA Preparation

Frozen whole blood in human were transferred to 1.0 mL of TRIzol reagent™ (Invitrogen, Carlsbad, CA).

Total RNA was isolated using TRIzol reagent™ (Invitrogen, Carlsbad, CA), according to protocols provided by the manufacturer's recommendations (http://www.protocol-online.org/). In brief, after homogenization with TRIZOL, 0.2 mL chloroform was added for each 1 mL of TRIZOL usage, and the sample was centrifuged at 12,000 × g, 4°C for 15 min.? Then, the aqueous phase was transferred into a new tube and 0.5 mL of isopropyl alcohol was added. After centrifugation at 12,000 × g, 4°C for 10 min, the RNA was precipitated as a white pellet at the bottom of the tube. The RNA pellets in 75% ethanol were washed and centrifuged at 7,500 × g, 4°C for 5 min. The RNA pellets were dried and dissolved in RNase-free water and incubated for 10 min. at 55 to 60°C. The total RNA was quantified using the NanoDropND -1000 Spectrophotometer (NanoDrop, Montchanin, USA). After quantification the total RNA was stored at −70°C. An aliquot of total RNA was resolved with the use of agarose gel electrophoresis and RNA integrity was assessed from a visual comparison of the relative intensities of the 18S and 28S rRNA bands. For all samples, the intensity of the 28S rRNA band exceeded that of the 18S band.

## cDNA Microarray

**cDNA Microarray** : A human cDNA microarray was primarily derived from a commercially available master set of approximately 15,000 human verified sequences (Research Genetics, Inc., Huntsville, AL). The 15,000-human cDNA clone set was sorted for a list of genes (1,152 elements) representing families involved in differentiation, development, proliferation, transformation, cell-cycle progression, immune response, transcription and translation factors, oncogenes, and molecules involved in cell growth and maintenance. PCR-amplified cDNAs were spotted on nylon membranes. The general methodology of arraying is based on the procedures of DeRisi *et al.*[10].

**cDNA Radiolabeling** : Total RNAs prepared from control and experimental group were used to synthesize $^{33}$P-labeled cDNAs by reverse transcription. Briefly, 3-10 g of RNA were labeled in a reverse transcription reaction containing 5 X first-strand PCR buffer, 1 g of 24-mer poly dT primer, 4 L of 20 mM each dNTP excluding dCTP, 4 L of 0.1 M DTT, 40 U of RNase inhibitor, 6 l of 3,000 Ci/mmol-$^{33}$P dCTP to a final volume of 40 L. The mixture was heated at 65°C for 5 min, followed by incubation at 42°C for 3 min. 2 L (specific activity: 200,000 U/mL) of Superscript II reverse transcriptase (Life Technologies, Inc., Rockville, MD) was then added and the samples were incubated for 30 min at 42°C, followed by the addition of 2 L of Superscript II reverse transcriptase and another 30 min of incubation. 5 L of 0.5 M EDTA was added to chelate divalent cations. After the addition of 10 L of 0.1 M NaOH, the samples were incubated at 65°C for 30 min to hydrolyze the remaining RNA. Following the addition of 25 L of 1 M Tris (pH 8.0), the samples were purified using Bio-Rad 6 purification columns (Hercules, CA). This resulted in $5 \times 10^6$ to $3 \times 10^7$ cpm per reaction[11].

**Human: Hybridization & Scanning** : cDNA microarrays were pre-hybridized in a hybridization buffer containing 4.0 mL Microhyb (Research Genetics), 10 L of 10 mg/mL human Cot 1 DNA (Life Technologies), and 10 L of 8 mg/mL poly dA (Pharmacia, Peapack, NJ). Both Cot 1 and poly dA were denatured at 95°C for 5 min prior to use. After 4 h of prehybridization at 42°C, approximately $10^7$ cpm/ml of heat-denatured (95°C, 5 min) probes were added and incubation continued for 17 h at 42°C. Hybridized arrays were washed three times in 2X SSC and 0.1% SDS for 15 min at room temperature. The microarrays were exposed to phosphoimager screens for 1-5 days, and the screens were then scanned in a FLA-8000 (Fuji Photo Film Co., Japan) at 50-m resolution[10].

## Data Analysis

The data were normalized with Z scores by subtracting each average of gene intensity and dividing with each standard deviation. A Z score represents the

variability from the average intensity, expressed in units of standard deviation, for each of the 684 genes. A Z score provides flexibility to compare different sets of microarray experiments by adjusting differences in hybridization intensities. Gene expression differences between microarray experiments were calculated by comparing Z score differences for the same genes in different microarrays, which allowed gene expression to be compared in different samples. Z differences were calculated by subtracting the Z score of the control from each Z score of the control sample. These Z differences were further normalized to distribute their position by dividing with the average z difference and dividing with the standard deviation of the z differences. These distributions represented Z ratio values and allowed efficient comparisons to be made between microarray experiments[11]. Scatter plots of intensity values were produced in Spotfire (Spotfire, Inc., Cambridge, MA)[12]. Color overlay images were produced in Adobe Photoshop 5.0 (Adobe Systems, Inc., San Jose, CA). Cluster analysis was performed on Z-transformed microarray data by using two programs available as shareware from Michael Eisen's laboratory (http://rana.lbl.gov). Clusterings of changes in gene expression were determined by using public domain Cluster based on pair wise complete-linkage cluster analysis[13]. Raw gene expression, log values, and Z scores were averaged and are expressed as means ± standard deviation. Raw gene expression, log values and Z scores were averaged and are expressed as means ± standard deviation.

### Statistical Analysis

Statistical analyses were performed using the SPSS Statistical Analysis System (Version 12.0). Data were expressed as means ± S.D or frequencies (percentage). All statistical tests were two-sided. Chi-square analysis was used to exam the distribution of age and smoking habits between groups, and Student's t-test was performed to determine the significant difference of urinary Naphthol between groups. Differences were considered significant at the level of $P < 0.05$.

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