# 野生型(AhR+/+)ならびにアリールハイドロカーボン受容体欠損(AhR-/-)マウス肝臓における2,3,7,8-Tetrachlorodibenzo-p-Dioxinによる遺伝子誘導プロファイル


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# Gene Expression Profile by 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin in the Liver of Wild-Type (AhR+/+) and Aryl Hydrocarbon Receptor-Deficient (AhR–/–) Mice

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ABSTRACT. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is one of the most toxic environmental pollutants that cause various biological effects on mammals. The purpose of our study was to identify the genes involved in hepatotoxicity and hepatocarcinogenesis caused by TCDD. C57BL/6 (AhR+/+, wild type) and B6.129-AhR<tm1Bra>/J (AhR–/–, knock out) mice were injected i.p. with a single treatment of TCDD at the dose of 100 μg/kg body weight. Relative liver weight was significantly increased at 72 hr after TCDD treatment without an apparent histopathological change in AhR+/+ mice ($p<0.05$). TCDD treatment also significantly increased activity of serum alanine aminotransferase in AhR–/– mice ($p<0.05$). The liver was analyzed for gene expression profiles 72 hr later. As compared with AhR–/– mice, the expression of 51 genes (>3-fold) was changed in AhR+/+ mice; 28 genes were induced, while 23 genes were repressed. Most of the genes were associated with chemotaxis, inflammation, carcinogenesis, acute-phase response, immune responses, cell metabolism, cell proliferation, signal transduction, and tumor suppression. This study suggests that the microarray analysis of genes in the liver of AhR+/+ and AhR–/– mice may help to clarify the mechanism of AhR-mediated hepatotoxicity and hepatocarcinogenesis by TCDD.

KEY WORDS: gene expression, liver, microarray, TCDD.

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Dioxins are a heterogeneous mixture of chlorinated dibenzo-*p*-dioxin and dibenzofuran (PCDD and PCDF) congeners. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is considered to be most toxic of the dioxin congeners [37, 43]. TCDD induces a variety of biological responses including induction of cytochrome P-4501A (CYP1A), reproductive and developmental defects, immunotoxicity, thymus atrophy, epothelial disorders, liver damage, wasting syndrome, and cancer [13, 26]. There is an overflow of data indicating that TCDD is a potent tumor promoter in rat and mouse liver and lung, as well as in mouse skin [20, 27, 31, 36]. TCDD causes tumor promotion by interfering with intracellular signal transduction pathways related to growth factors and cytokines such as transforming growth factor (TGF) and interleukin-1β (IL-1β) [18, 20, 24, 27, 31, 36, 42]. In addition, TCDD exposure results in reactive oxygen species production and an oxidative stress response in adult and fetal tissues of experimental animals. The reactive oxygen species may in turn oxidize DNA bases, leading to strand breakage or clastogenic effects [31]. Nevertheless, the mechanism of TCDD-induced carcinogenesis is incompletely understood.

TCDD binds to the cytosolic AhR, cytosolic ligand-activated transcription factor. This receptor has the potential to up-regulate and down-regulate the expression of a large number of genes with diverse functions, including those of the Ah gene battery, such as CYP1A1 and CYP1A2 [18, 24, 42]. Activation of the AhR is clearly associated with a cellular oxidative stress response, mediated in part by the induction of cytochrome P450 [31]. AhR is widely expressed in mammalian tissues, and it is hypothesized that initial binding to the AhR is linked to the broad spectrum of biochemical and toxic responses observed in laboratory animals and cells exposed to TCDD and other halogenated aromatic contaminants that bind the AhR [27]. Animal experiments revealed that lipophilic TCDD accumulated mostly in the liver and to less extent in fat tissue through absorption from intestine [18]. Concerning the liver, epidemiologic studies in accidently exposed populations revealed hepatotoxicity, and chronic TCDD treatment promoted liver tumor formation in laboratory animals [18]. TCDD has also proved to be positive in cell transformation assays in cultured rodent and human cells [18]. Although several previous studies analyzed the gene expression profiles of hepatocyte cell line cells treated with TCDD using cDNA microarray [10, 42] and gene expression profiling approach to *in vivo* material using serial analysis of gene expression (SAGE) [18], there are few reports on the expression of specific reactive genes against hepatotoxicity of TCDD.

Therefore, in this study to identify the specific genes involved in hepatotoxicity and hepatocarcinogenesis induced by TCDD, we analyzed the differences of gene expression profile in the liver of AhR+/+ and AhR–/– mice.

## MATERIALS AND METHODS

*Chemical*: TCDD (purity > 99%) was purchased from

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Cerilliant Cambridge Isotope Laboratory, Inc. (Andover, MA, U.S.A.). TCDD was dissolved in acetone and diluted in corn oil as described [15].

*Mice*: 10-week-old C57BL/6 male mice (AhR+/+) and B6.129-Ahr$^{tm1Bra}$/J (AhR–/–) mice were purchased from The Jackson Laboratory (Bar Harbor, MA, U.S.A.) and were allowed 2 weeks for acclimatization. The mice were housed in polyethylene cages containing wood shavings and were given rodent chow and water *ad libitum*. Mice were housed in rooms maintaining temperature of 21 ± 1°C, humidity of 55 ± 5%, and a 12-hr light/dark cycle. The experimental protocols were conducted in accordance with internationally accepted principles for laboratory animal use and care as found in the Korea Food and Drug Administration guidelines.

*TCDD treatment and sample collection*: Three mice of each treatment group were injected i.p. with TCDD at the dose of 100 μg/kg body weight. Control mice received the vehicle alone. At 72 hr after TCDD treatment, blood samples were drawn from the animals at necropsy and activities of alanine aminotransferase (ALT) and aspartate amino transferase (AST) were measured using a clinical chemistry analyzer (Bayer ADIVIA-120 hematology system, Tarrytown, NY, U.S.A.). The terminal body weights and liver weights were recorded. The left lateral lobe of the liver was processed for histopathologic evaluation with H&E staining. Three small pieces of liver (approximately 20 mg) from each mouse were stored at –20°C until use of RNA extraction (Ambion Inc, Austin TX, U.S.A.).

*Extraction of total RNA*: Each sample was placed into 1 m*l* of Trizol solution (Invitrogen, Carlsbad, CA, U.S.A.) and homogenized with a polytron homogenizer (Wheaton, Millville, NJ, U.S.A.). Total RNA was separated with Qiagen RNeasy mini kit, according to the protocol described by the manufacturer (Qiagen, Valencia, CA, U.S.A.). The RNA quality was assessed by analyzing the A260/A280 ratio (1.8 or above) and by evaluating the integrity of the 28S and 18S RNA bands using an Agilent 2100 Bioanalyzer (Agilent Technology, Palo Alto, CA, U.S.A.).

*Microarray analysis*: First- and second-strand cDNA synthesis, biotin-labeled cRNA synthesis, fragmentation of cRNA and hybridization reactions were performed as a customer service by Affymetrix Inc. (Santa Clara, CA, U.S.A.) and detailed descriptions were found at the Web site, http://www.affymetrix.com. Briefly, cDNA was synthesized using an one-cycle cDNA synthesis kit from 10 μg of each RNA sample. Labeled cRNA was synthesized from cDNA using a GeneChip IVT labeling kit according to the manufacturer's instructions. Approximately 20 μg cRNA was then fragmented in a solution of 5 × fragmentation buffer and RNase-free water at 94°C for 35 min. Labeled cRNA was hybridized to the GeneChip Test 3 array (Cat. No. Affymetrix 900341) to verify the quality of labeled cRNA. Then cRNA was hybridized by filling with appropriate volume of the clarified hybridization cocktail to the Mouse genome 430A 2.0 array (Cat. No. Affymetrix 900499). The cRNA was hybridized for 16 hr at 45°C in the hybridization oven 640 set to 60 rpm. After hybridization, the cocktail from the probe array was removed. Then, the probe array was completely washed with the appropriate volume of non-stringent wash buffer and stained with streptavidin phycoerythrin (SAPE) using GeneChip fluidics station 450. The probe array was scanned after the wash and staining protocols with GeneChip Scanner 3,000.

*Data analysis and clustering algorithm*: For each of the approximately 39,000 genes on the Affymetrix Mouse genome 430A 2.0 array, the data of induction or repression values were analyzed using Affymetrix software analysis (GCOS and DMT). Cluster analysis for gene expression was performed using Cluster 2.1.1 and 'TreeView' version 1.60 software supplied by Stanford University. The clustering was hierarchical using correlation distance as the measurement.

## RESULTS

*TCDD-induced liver damage*: In wild type AhR+/+ mice, hepatomegaly was reflected by statistically significant increases in relative liver weights at 100 μg/kg body weight (Fig. 1). The relative liver weight in AhR+/+ mice was increased by about 12.0% compared with the control. However, the relative liver weight in AhR –/– mice was apparently unaffected. As compared with the control, the activity of ALT in AhR–/– mice was significantly ($p<0.05$) increased but that of AST was not significantly changed in both AhR+/+ and AhR–/– mice (Fig. 2). The treatment of TCDD at the dose of 100 μg/kg body weight caused little change in histological examination after 72 hr in the liver of both AhR+/+ and AhR–/– mice (figure not shown).

*TCDD-induced gene expression changes in liver*: Microarray analysis was done to determine the hepatic gene expression 72 hr after treatment with TCDD at the dose of 100 μg/kg body weight in AhR+/+ and AhR–/– mice. As

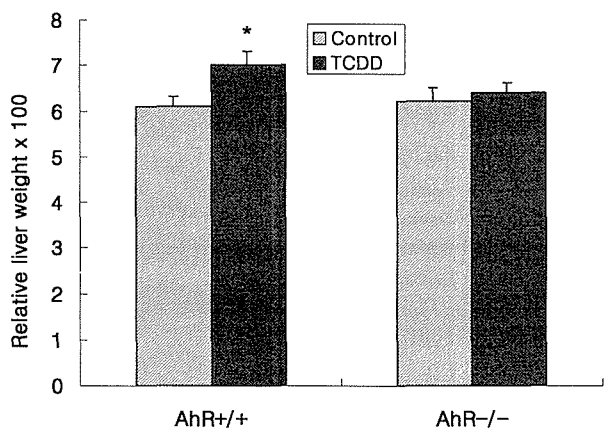


Fig. 1. Effects of TCDD on the relative liver weight in AhR+/+ and AhR–/– mice. Relative liver weight (%) was reported as a proportion of total body weight at 72 hr after TCDD treatment at the dose of 100 μg/kg body weight. Bars represent mean ± SD (n=3). *Significantly different from the control at $p<0.05$.

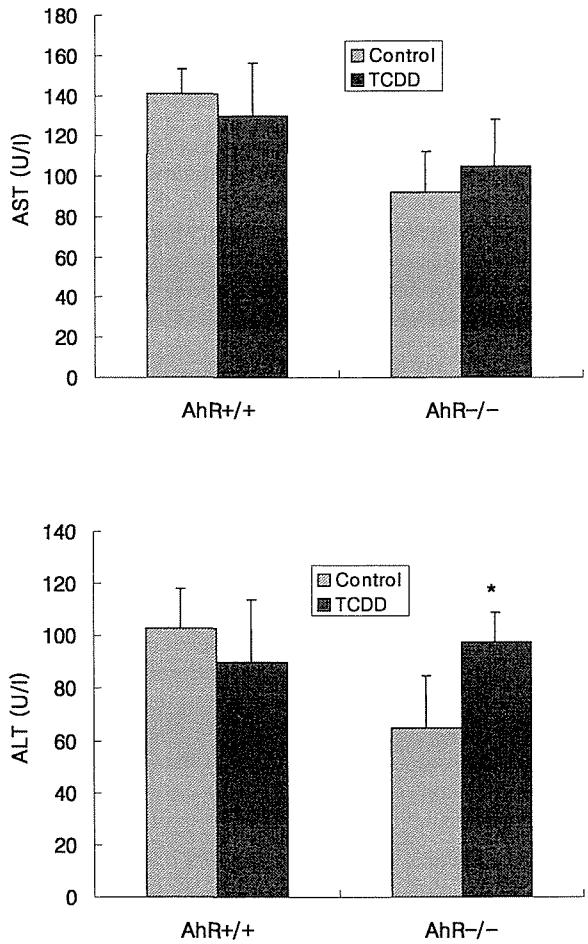


Fig. 2. Effects of TCDD on aspatate aminotransferase (AST) and alanine aminotransferase (ALT) levels in the serum of AhR+/+ and AhR−/− mice. Blood of mice was collected via retroorbital sinus at 72 hr after TCDD treatment at the dose of 100 μg/kg body weight. Bars represent mean ± SD (n=3). *Significantly different from the control at $p<0.05$.

compared with AhR−/− mice, the expression of 51 genes (>3-fold) was changed in AhR+/+ mice (Table 1); 28 genes were induced, while 23 genes were repressed (Table 2). The genes intensively up-regulated in only AhR+/+ mice compared with AhR−/− knockout mice were Mcsp, Myc, Hspa2, Atf3, Plcb2, S100a8, Ngp, Saa2, S100a8, S100a9, Cyp4f16, Tnfrsf1b, Csf2rb2, Plcb2,Saa2, Adamdec1,Csf2rb2, Cdgap, H2-D1, Cml5, Kcnq2, and Meig1 (Table 1). Meanwhile, the genes intensively down-regulated in only AhR+/+ mice as compared with AhR−/− mice were Slc13a2, Afmid, Csad, 1810073K19Rik, E130112L23Rik, Upk3b, Vampl, Tieg1, Erbb2ip, Ngfa, Cdc20, Cabyr, and Lect1 (Table 2).

## DISCUSSION

To identify the specific genes related with AhR-mediated TCDD hepatotoxicity we analyzed the differences of liver gene expression induced by TCDD at the dose of 100 μg/kg body weight in AhR+/+ and AhR−/− mice. We selected a 72 hr time-point for a variety of reasons. This time point would provide for a complete gene induction response within the liver without the complication of a significant secondary inflammatory response (cellular infiltration) or fibrosis likely to be encountered at later time points [38]. Although histopathological evaluation was performed in our study, apparent lesions in the liver were not observed. Meanwhile, in our preliminary study the treatment of TCDD at the dose of 150 μg/kg body weight caused an increase in the number of apoptotic cells and inflammatory infiltrates in the liver of C57BL/6 mice. In this study, TCDD treatment with 100 μg/kg body weight resulted in a significant increase in the relative liver weight at 72 hr but no histopathological changes were observed. These results are consistent with a recent study that reported increases in liver weights but no alterations in body weight after a single oral dose of TCDD at the concentration up to 100–300 μg/kg [4].

In our study, the levels of enzymes decreased at 72 hr after TCDD treatment in AhR+/+ mice, while increased in AhR−/− mice. Fletcher *et al.* [9] also reported that TCDD treatment caused a decrease in ALT at day 7 in male Sprague-Dawley rats. Meanwhile, Boverhof *et al.* [4] reported that a significant treatment related alteration were noted in ALT and the levels increased steadily after 24 hr to a maximum of 2.6-fold at 168 hr, indicative of mild liver injury in TCDD-treated immature ovarectomized female mice.

In our study, despite the absence of apparent histopathological lesions after treatment with TCDD, changes of gene expression that might be indicative of changes in cellular function were observed. Because of the placement of chlorine atoms on the molecule, TCDD resists metabolic processing and it persists within the cell and produces sustained alterations in gene expression [28, 40]. We suspect that persistence of TCDD is an important factor in producing adverse effects. In our study, we compared the differences of liver gene expression induced or repressed by TCDD treatment between AhR+/+ and AhR−/− mice. The functions of 28 genes intensively up-regulated (>3-fold) in only AhR+/+ mice compared with AhR−/− knockout mice were associated with maintenance and stabilization of spermatozoa mitochondria (Mcsp) [1], cell proliferation and transformation related with carcinogenesis (Myc) [14, 17], stress response (Hspa2, Atf3, Plcb2) [2, 11], chemotaxis (S100a8) [35], inflammatory response (Ngp, Saa2, S100a8, S100a9, Cyp4f16, Tnfrsf1b, Csf2rb2, Plcb2) [6, 34, 35], acute-phase response (Saa2) [34] and immune response (Adamdec1,Csf2rb2, Cdgap, H2-D1) [3, 41], cell adhesion (Cml5) [25], neuronal excitation (Kcnq2) [8], cell division (Meig1) [33]. Meanwhile, the functions of 23 genes intensively down-regulated (>3-fold) in only AhR+/+ mice were associated with cell metabolism (Slc13a2, Afmid, Csad, 1810073K19Rik, E130112L23Rik, Upk3b) [16, 19, 23, 30], nerve regeneration (Vampl) [5], cell growth (Tieg1, Erbb2ip, Ngfa) [21, 22], cell cycle (Cdc20) [32], testis-specific $Ca^{2+}$-binding protein (Cabyr) [29] and inhibition of

Table 1. Major genes induced at 72 hr after treatment of TCDD at the dose of 100 μg/kg body weight in AhR+/+ mice in comparison with AhR–/– mice

| Probe Set ID | Gene Title | Gene Symbol | Fold change |
|---|---|---|---|
| 1417101_at | heat shock protein 2 | Hspa2 | 3.15 |
| 1417944_at | guanine nucleotide binding protein, gamma 4 subunit | Gng4 | 4.38 |
| 1418358_at | mitochondrial capsule selenoprotein | Mcsp | 4.26 |
| 1418722_at | neutrophilic granule protein | Ngp | 3.07 |
| 1419075_s_at | serum amyloid A 2 | Saa2 | 3.39 |
| 1419203_at | gene trap locus F3a | Gtlf3a | 3.99 |
| 1419394_s_at | S100 calcium binding protein A8 (calgranulin A) | S100a8 | 5.36 |
| 1419476_at | ADAM-like, decysin 1 | Adamdec1 | 3.1 |
| 1420800_a_at | potassium voltage-gated channel, subfamily Q, member 2 | Kcnq2 | 3.11 |
| 1422988_at | N-sulfoglucosamine sulfohydrolase (sulfamidase) | Sgsh | 3.32 |
| 1423410_at | meiosis expressed gene 1 | Meig1 | 3.53 |
| 1424811_at | camello-like 5 | Cml5 | 3.04 |
| 1424942_a_at | myelocytomatosis oncogene | Myc | 4.35 |
| 1426597_s_at | expressed sequence C79267 | C79267 | 3.34 |
| 1430172_a_at | cytochrome P450, family 4, subfamily f, polypeptide 16 | Cyp4f16 | 3.19 |
| 1430173_x_at | cytochrome P450, family 4, subfamily f, polypeptide 16 | Cyp4f16 | 3.01 |
| 1441115_at | DNA segment, Chr 18, ERATO Doi 232, expressed | D18Ertd232e | 3.09 |
| 1448296_x_at | | | 3.33 |
| 1448756_at | S100 calcium binding protein A9 (calgranulin B) | S100a9 | 6.4 |
| 1448951_at | tumor necrosis factor receptor superfamily, member 1b | Tnfrsf1b | 3.93 |
| 1449233_at | muscle, intestine and stomach expression 1 | Mist1 | 3.31 |
| 1449360_at | colony stimulating factor 2 receptor, beta 2, low-affinity | Csf2rb2 | 3.71 |
| 1449363_at | activating transcription factor 3 | Atf3 | 3.87 |
| 1450162_at | D4, zinc and double PHD fingers, family 3 | Dpf3 | 3.01 |
| 1450255_at | Cdc42 GTPase-activating protein | Cdgap | 3.07 |
| 1450412_at | transducin (beta)-like 2 | Tbl2 | 3.24 |
| 1452481_at | phospholipase C, beta 2 | Plcb2 | 3.1 |
| 1452544_x_at | histocompatibility 2, D region locus 1 | H2-D1 | 3.31 |

Table 2. Major genes repressed at 72 hr after treatment of TCDD at the dose of 100 μg/kg body weight in AhR+/+ mice in comparison with AhR–/– mice

| Probe Set ID | Gene Title | Gene Symbol | Fold change |
|---|---|---|---|
| 1416029_at | TGFB inducible early growth response 1 | Tieg1 | –3.04 |
| 1417867_at | adipsin | Adn | –4 |
| 1418174_at | D site albumin promoter binding protein | Dbp | –3.2 |
| 1418857_at | solute carrier family 13, member 2 | Slc13a2 | –3.15 |
| 1419857_at | | | –3.11 |
| 1420942_s_at | | | –3.02 |
| 1421862_a_at | vesicle-associated membrane protein 1 | Vamp1 | –3.09 |
| 1423257_at | | | –7.73 |
| 1427047_at | nucleoporin 188 | Nup188 | –3.33 |
| 1427981_a_at | cysteine sulfinic acid decarboxylase | Csad | –3.1 |
| 1424558_a_at | calcium-binding tyrosine-(Y)-phosphorylation regulated | Cabyr | –3.81 |
| 1431722_a_at | arylformamidase | Afmid | –3.33 |
| 1436643_x_at | RIKEN cDNA 1810073K19 gene | 1810073K19Rik | –3.03 |
| 1436768_x_at | RIKEN cDNA E130112L23 gene | E130112L23Rik | –3 |
| 1438211_s_at | D site albumin promoter binding protein | Dbp | –3.69 |
| 1439028_at | | | –3.3 |
| 1439079_a_at | Erbb2 interacting protein | Erbb2ip | –3.08 |
| 1439394_x_at | cell division cycle 20 homolog (S. cerevisiae) | Cdc20 | –3.09 |
| 1450222_x_at | nerve growth factor, alpha | Ngfa | –4.07 |
| 1452183_a_at | GTL2, imprinted maternally expressed untranslated mRNA | Gtl2 | –7.17 |
| 1454881_s_at | uroplakin 3B | Upk3b | –3.64 |
| 1456624_at | DNA segment, Chr 11, ERATO Doi 498, expressed | D11Ertd498e | –3.05 |
| 1460258_at | leukocyte cell derived chemotaxin 1 | Lect1 | –3.88 |

tumor growth (Lect1) [12].

The results reported here suggest that toxicity may reflect sustained alterations in the expression of many genes and that the changes reflect both direct and indirect effects of

TCDD. Recently, several groups reported gene expression profiles *in vitro* and *in vivo* models exposed to TCDD. Using cDNA microarray technology, Frueh *et al.* (2001) reported that numerous induced genes of human liver HepG2 cell by TCDD treatment appear to have functions in cell growth and proliferation, cell adhesion, antioxidative activity, acute-phase responses, and these results may be associated with maintaining homeostasis against TCDD exposure. Using pathway-specific cDNA arrays to detect the transcriptional signature induced by TCDD in C57BL/6 mice after intraperitoneal injection with 50 μg/kg body weight of TCDD, TCDD altered the expression of a large array of genes involved in apoptosis and angiogenesis [42]. The other experiment using serial analysis of gene expression (SAGE) technique revealed that in C57BL/6 mice the genes involved in hepatotoxicity and hepatocarcinogenesis induced by TCDD treatment with a single oral dose of 20 μg/kg body weight were not only the genes encoding drug metabolizing enzymes and stress response genes but also a wide variety of genes encoding cytoskeleton related proteins, signal transduction, and plasma proteins [18]. Regarding the liver genes involved in xenobiotics metabolism or stress response, signal transduction, cell cycle and cell proliferation, the expression profiles of these studies were mostly consistent with our results obtained by using the oligonucleotide DNA microarray chip (Affymetrix) *in vivo* mouse model.

In our study, the most significant gene changed by TCDD in both AhR+/+ and AhR–/– knockout mice was Cyp1a1 which was up-regulated over 100-fold by TCDD, although the change was not shown in our comparative data, indicating that the difference from change in gene expression by TCDD between AhR+/+ mice and AhR–/– mice was less than 3-fold. Most compounds that are known to induce Cyp1a1 have been shown be the ligand for the AhR [39]. However, there are reports that Cyp1a1 induction can be seen with compounds that are not apparent AhR ligands based on their inability to compete with TCDD for receptor binding [39]. In addition, there are different hypotheses proposed for how Cyp1a1 could be induced by mechanisms that do not involve in the AhR [7]. Based on the similarity of effect of TCDD on Cyp1a1 expression of both AhR+/+ and AhR–/– mice, it is likely that there are other signaling pathways for Cyp1a1 induction though more studies would be required to conclusively understand those.

Overall, the results of our study imply that cellular responses to TCDD is notably complex and is associated with alterations in the expression of a large array of genes, and can provide a fingerprint genes that may help to clarify the mechanism of TCDD effects on hepatic genotoxicity and carcinogenesis.

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