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CYP1A1 and MT1K are congener specific biomarker genes for liver diseases induced by PCBs

Sisir K. Dutta [a,*], Somiranjan Ghosh [a], Supriyo De [a], Eric P. Hoffman [b]

[a] *Molecular Genetics Laboratory, Department of Biology, Howard University, 415 College Street, NW, Washington DC 20059, USA*
[b] *Center for Genetic Medicine, Children's National Medical Hospital, 111 Michigan Avenue, NW, Washington DC 20010, USA*

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Abstract

We have identified two most significant biomarker genes, CYP1A1 (69.81 up-regulation) and MT1K (14.66 up-regulation), showing highest overexpression at p-value <0.005. These were selected out of several hundred genes induced *in vitro*, using PCB exposed human liver (HepG2) cells. Over expression of the CYP1A1 (cytochrome P450) gene was specific to PCB-77 and MT1K (Metallothionein) to PCB-153. Affymetrix oligonucleotide microarrays (mRNA) were used to screen the entire genome of human liver cells in a time-dependent exposure and were further validated by quantitative real-time RT-PCR.

Keywords: PCB-77; PCB-153; Biomarker genes; CYP1A1; MT1K

1. Introduction

Almost all human diseases involve gene-environment interaction. Polychlorinated biphenyls are widespread persistent residual environmental contaminants, which have been widely used for various industrial applications. The major source of PCB intake in the general population is via oral ingestion of contaminated food products. Due to their structural differences (coplanar and non-coplanar) the modes of action of these PCBs are also different, leading to diseases following differential gene expression. Coplanar PCB and related congeners (e.g., 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)), induce gene expression by a ligand-dependent transactivating factor, the arylhydrocarbon receptor, and alternative pathways for gene expression (e.g., c-Src and cross linked with the MAP kinase pathway (Aoki, 2001)). Human genome-wide gene expression has revealed specific biomarkers for Huntington disease (Borovecki et al., 2005). These stable genomic biomarkers should enable us to make early diagnoses of diseases in humans caused by hazardous chemicals in the environment, such as PCBs.

PCB exposure has been associated with various diseases, causing alteration of gene expression, and has also been strongly implicated in degenerative disease associated with genomic instability, carcinogenesis, and aging (Slane et al., 2006). Specifically, the current hypothesis is that mitochondrial production of reactive oxygen species (ROS) (i.e. superoxide and hydrogen peroxide), causes oxidative stress during PCB exposure, and this increased production of ROS contributes to biological effects of PCBs on cell growth in prostate and breast epithelial cells. The non-*ortho*-polychlorinated biphenyl (PCB) congener 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) can uncouple the catalytic cycle of fish (scup) cytochrome P4501A (CYP1A) and mammalian (rat, human) CYP1A1, stimulating release of reactive oxygen species. PCB-77 also inactivates CYP1A in an NADPH-, oxygen- and time-dependent process, linked to uncoupling (Schlezinger et al., 2006). Metallothionein (MT1K) is a cysteine-rich, metal binding protein that can be induced by a variety of agents. We report here the molecular evidence of these two genes that are excellent candidate genomic biomarkers for detecting congener specific PCB-induced human liver diseases.

2. Materials and methods

2.1. PCB exposure in human liver cells

In this study, we selected a metabolically competent human hepatocellular carcinoma cell line (HepG2), which retains many of the functions of normal liver cells (Knowles et al., 1980) and expresses the activities of several phases I and II xenobiotic metabolizing enzymes (Knasmuller et al., 1998). Human liver (HepG2) cells were grown in DMEM medium according to ATCC (Manassas, VA) protocol. The human liver (HepG2) cells were plated in 100 mm tissue cul-

* Corresponding author. Tel.: +1 202 806 6942; fax: +1 202 806 5832.
E-mail address: sdutta@howard.edu (S.K. Dutta).

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ture dishes in their respective media. Cultures were confluent after 4–5 days, and all experiments were performed with confluent monolayers. Medium was refreshed prior to the experiment. PCB-153 and PCB-77 (dissolved in DMSO) were added to each plate individually according to their LD_{50} (70 μM) concentrations (Chen et al., 2006; De et al., 2006), where the final concentration of DMSO was ≤0.1%. The exposure was the same in all the experiments, where 0-h exposure serves as the control.

2.2. RNA isolation and microarray analysis

Total RNA from the cells after different exposure levels was extracted by using Trizol reagent (Invitrogen). The quality of total RNA was analyzed by using the RNA 6000 Nano LabChip kit on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). The RNA extracted was then reverse transcribed to cDNA with an oligo-dT primer containing T7 RNA polymerase promoter. The cDNA was then used as a template for *in vitro* transcription using the ENZO BioArray RNA transcript labeling kit (Affymetrix, CA). Biotin-labeled cRNA was purified, then fragmented randomly to approximately 200 bp (200 mM Tris–acetate, pH 8.2, 500 mM KOAc, 150 mM MgOAc) prior to hybridizing to Affymetrix Human Genome Array for 16 h. The microarray was washed and stained, and fluorescent images were obtained using the Affymetrix 3000 Scanner. Quality control measures included >4-fold cRNA amplification (from total RNA/cDNA), scaling factors <2 to reach a whole-chip normalization of 800, and visual observation of hybridization patterns for chip defects, following the protocols of Zhao and Hoffman (2004) for quality control.

2.3. Gene expression analysis

We have used a large variety of statistical methods available in GeneSpring. Briefly, probe-set analysis results derived from Microarray Suite version 5.0 and dChip (Seo et al., 2006) were used for preliminary image analysis as follows. Genes with 10% present calls (P) were selected for further analysis. Hierarchical clustering analysis were limited to probe sets with at least 10% present calls and p-value <0.05 for the Welch t-test, corrected with Benjamini and Hochberg False Discovery Rate between any two time points. A hierarchical clustering algorithm using the Pearson correlation was then used to temporally group those probe sets based on their expression patterns across the five time points. Besides GeneSpring, HCE clustering, which permits different computational algorithms and stringencies of the analysis, was employed in an interactive manner (Seo and Hoffman, 2006). Temporal clusters of genes that are specific and shared between the two disease stages, prioritized based upon a consideration of combined support from p-value from both dChip and MAS5.0 (GeneSpring), and visual analysis (HCE), were superimposed on gene ontology flow charts from the BayGenomics Programs in Genomic Applications (GenMAPP) (Dahlquist et al., 2002) and pathway-assist software (Stratagene, CA).

2.4. Quantitative real-time RT-PCR analysis

The differential expression of these genes was confirmed by quantitative RT-PCR, and the expression pattern of these genes was determined by *in situ* hybridization. For true validation, the HepG2 cells were grown again in triplicates with the addition of 70 μM of PCB-77 and PCB-153 respectively, exactly as in the cell culture for microarray analysis. RNA was extracted using the Trizol-Chloroform method and purified with the Qiagen RNeasy kit. The extracted RNA was then quantified spectrophotometrically using the 'Nanodrop' instrument at 260 nm wavelength. The RNA was then diluted to 300 ng/ml. For cDNA synthesis, reverse transcriptions were done according to the Invitrogen protocol. QRT-PCR was performed with the SYBR green method by using the MyiQ Single-Color RT-PCR detection system (Bio-Rad). Primers (forward and reverse) were designed with the PRIMER3 program (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and the sequence was as follows: CYP1A1 (forward), AAACAGGGCCACATAGATGC; CYP1A1 (reverse), AGGGTCCTGGTTTGGCTAGT; MT1K (forward), TAAGCTTGCGACCGCTTAAT; MT1K (reverse), GGAACAGCTCTTCTCCCAGA, GAPDH (forward), GCAGTGGCTCATGGTTTACA; GAPDH (reverse), CAAAGCACCCGGTTACTCAT; 28S (forward) AAACTCTGGTGGAGGTCCGT; 28S (reverse), CTTACCAAAAGTGGCCCACTA. Initial analyses were performed by using the ICYCLER system software (Bio-Rad). Relative gene expressions were calculated by using the $2^{-\Delta\Delta Ct}$ method, in which Ct indicates cycle threshold, the fractional cycle number where the fluorescent signal reaches detection threshold. GAPDH was used as an internal control for tissue samples and 28S rRNA as an internal control.

3. Results

Gene expression profiling with PCB-153, followed by probe-level analysis with GCOS, compared with analysis with dChip, showed that there were 983 significant genes in GCOS compared to 1787 genes from dChip with 606 genes in common (61.6%). Similarly, comparison with PCB-77 showed that there were 414 significant genes in GCOS compared to 645 genes from dChip with 272 genes in common (65.7%). Another com-

Table 1
Comparative list of the top five (5) genes which have been reported to have disease connections are shown in this table

Gene symbol	Gene ID	Gene name	Fold change (up-regulation)	Remarks[a]
A. List of gene expression by PCB-153 in HepG2 cells *in vitro*				
217546_at	MT1K	Metalothionein 1K	14.66	Polycystic liver disease (confirmed by RT-PCR)
201008_s_at	TXNIP	Thioredoxin interacting protein	11.91	Polycystic liver disease
229800_at	DCAMKL1	Doublecortin and CaM kinase-like 1	9.79	–
210472_at	MT1G	Metalothionein 1G	8.47	Polycystic liver disease
205749_at	CYP1A1	Cytoxhrome P450, family 1A1	6.70	Fatty liver, hepatocellular carcinoma, fibrosis, cirrhosis (confirmed by RT-PCR)
B. List of gene expressions by PCB-77 in HepG2 cells *in vitro*				
205749_at	CYP1A1	Cytoxhrome P450, family 1A1	69.81	Fatty liver, hepatocellular carcinoma, fibrosis, cirrhosis (confirmed by RT-PCR)
205681_at	BCL2A1	BCL-2 related protein A1	9.89	
228967_at	SUI1	Putative translation initiating factors	8.30	Chronic active liver disease
207097_at	SLC17A2	Solute carrier family 17 A2	6.69	Polycystic liver disease
209921_at	SLC7A11	Solute carrier family 7, member 11	5.94	Polycystic liver disease

Out of 188 and 125 genes up-regulated by PCB-153 and PCB-77 respectively, the data represent the top five (5) genes whose fold changes increased more that 5.5 with a p-value <0.001. The marked fold change (highest) differences in CYP1A1 by PCB-153 (6.70) and by PCB-77 (69.81), MT1K (14.66) by PCB-153 led us to select them biomarker genes for liver disease *in vitro*, which is also validated by real-time RT-PCR (Fig. 1). No significant MT1K gene expression was observed when HepG2 Cells were treated with PCB-77.

[a] Disease connections are adapted after National Toxicology Program (2006), Slane et al. (2006), Lynes et al. (1993), and Ganguly et al. (1996).