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Transcriptional profiling and biological pathway analysis of human equivalence PCB exposure *in vitro*: Indicator of Disease and disorder development in humans

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Abstract

Background and Aims—Our earlier gene-expression studies with a Slovak PCBs-exposed population have revealed possible disease and disorder development in accordance with epidemiological studies. The present investigation aimed to develop an *in vitro* model system that can provide an indication of disrupted biological pathways associated with developing future diseases, well in advance of the clinical manifestations that may take years to appear in the actual human exposure scenario.

Methods—We used human PBMC (Primary Blood Mononuclear Cells) and exposed them to a mixture of human equivalence levels of PCBs (PCB-118,138,153,170,180) as found in the PCBs-exposed Slovak population. The microarray studies of global gene expression were conducted on the Affymetrix platform using Human Genome U133 Plus 2.0 Array along with Ingenuity Pathway Analysis (IPA) to associate the affected genes with their mechanistic pathways. High-throughput qRT-PCR Taqman Low Density Array (TLDA) was done to further validate the

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The contents of this report are solely the responsibility of the authors.

Conflict of Interest

There is no conflict of interest among the authors in the present work.

Author's Contribution

SG developed the work, design and performed the *in vitro* experimental work, IPA Analysis, and also wrote the manuscript. PM performed the statistical analysis of the microarray results. SGM ran the microarray experiments. TT, LM, ES were responsible for providing the epidemiological information on the human subjects, included herein. SZ provide valuable information towards this study. EH, CL and SKD provided support and direction to the manuscript. SKD held the NIEHS/UO1 and R200174 Grant.

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selected 6 differentially expressed genes of our interest, viz., ARNT, CYP2D6, LEPR, LRP12, RRAD, TP53, with a small population validation sample (n=71).

Results—Overall, we revealed a discreet gene expression profile in the experimental model that resembled the diseases and disorders observed in PCBs-exposed population studies. The disease pathways included Endocrine System disorders, Genetic disorders, Metabolic diseases, Developmental disorders, and Cancers, strongly consistent with the evidence from epidemiological studies.

Interpretation—These gene finger prints could lead to the identification of populations and subgroups at high risk for disease, and can pose as early disease biomarkers well ahead of time, before the actual disease becomes visible.

Keywords

PCBs; Human PBMC; Gene expression; Taqman Low-density array (TLDA); Pathway Analysis; Disease and Disorders; Biomarkers

1. Introduction

Even after the production of Polychlorinated biphenyls (PCBs) was banned in the 1970s, more than a billion kilograms were produced (Erickson, 1988), and they remain persistent and ubiquitous environmental contaminants that are routinely found in samples of human and animal tissues (Giera et al, 2011; Yang et al, 2010). Improper disposal of PCBs has been a major source of environmental contamination. Subsequent human exposure has been associated with toxic effects on various organs including the nervous, reproductive, and immunologic systems. The exposures to PCBs in a highly exposed Slovak population were associated with endocrine disorders (Radiková et al, 2008), diabetes (Ukropec et al, 2010), and reproductive (Plísková et al., 2005), neurological (Park et al., 2009, 2010), and hearing impairments (Trnovec et al., 2010), in addition to cancers (Pavúk et al., 2003, 2004, Bencko et al., 2009), and immunotoxicity (Horváthová et al., 2011a, b). Recent evidence suggests that exposure to some commonly encountered environmental contaminants, e.g. organochlorine compounds (OC; including several PCB congeners and chlorinated pesticides) may also contribute to Type 2 diabetes (Longnecker & Daniels, 2001, Carpenter 2008, Dirinck et al., 2011, Lee et al., 2011). There is growing evidence that perturbations of central endocrine regulatory systems by the endocrine disrupting chemicals (EDCs; e.g. Dioxins, PCBs, OC, etc.) established in early gestation may contribute to the development of obesity in later life (Alonso-Magdalena et al., 2011, Wang et al., 2008, Turyk et al., 2009a,b, Philibert et al., 2009).

The “developmental basis of disease” hypothesis posits that even seemingly minor exposures during early development can lead to functional deficits and increased disease risks later in life. However, it would be difficult to follow humans for decades to see if they develop diseases based on what they were exposed to before birth. On the other hand, researchers are now able to use new technologies to examine gene expression changes in tissues during development and link them to the pathogenetic pathways known to mediate the onset of disease later in life. Therefore, our study is based on the idea that changes in gene expression of a defined panel of genes can serve as both a robust biomarker of

exposure to a group of compounds and as an indicator for future risk for specific diseases. Our earlier studies revealed that different PCB congeners (due to its structural differences) play a critical role in the mode(s) of action *in vitro* that changes the important cellular and signaling process and their potential to cause disease and developmental disorders (Dutta et al., 2008, Ghosh et al., 2011), including studies of gene expression in PCB-exposed children (Dutta et al., 2012, Mitra et al., 2012). The present study is designed to categorize some putative biomarkers through *in vitro* studies, indicating the affected molecular mechanisms and specific pathways that can be of predictive value of future risks of developing disease following an exposure event to PCBs.

2. Methods

2.1 Chemicals

PCB-118 (2,3',4,4',5-pentachlorobiphenyl, Product # RPC-106, CAS # 031508-00-6), PCB-138 (2,2',3,4,4',5'-Hexachlorobiphenyl) (Product # RPC-088, CAS # 35065-28-2), PCB-153 (2,2',4,4',5,5'-hexachlorobiphenyl) (Product #RPC-047, CAS # 35065-27-1), PCB-170 (2,2', 3, 3', 4, 4', 5-heptachlorobiphenyl, Product # RPC-110, CAS# 035065-30-6), and PCB-180 (2,2',3,4,4',5,5'-heptachlorobiphenyl, Product # RPC-094, CAS # 035065-29-3) with a purity of >97.1 – 99.0 ±0.5% used herein are products of Ultra Scientific (North Kingstown, RI, USA). Dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO) was used for dissolving PCBs. A 2 ng/μl stock solution of the PCBs was prepared in DMSO to the working concentrations, in the same diluents. RPMI 1640 and Fetal Bovine Serum (FBS, Heat Inactivated, US Origin), Penicillin/Streptomycin were obtained from Invitrogen (Carlsbad, CA, USA). Phytohemagglutinin-M (PHA-M) was from Roche Diagnostics GmbH (Madison, WI, USA). Pokeweed Mitogen was from Life Technologies (Carlsbad, CA., USA). Trizol reagent from Invitrogen Corp. (Carlsbad, CA, USA) was used for RNA extraction. For microarray, GeneChip® Human Genome HU133 Plus 2.0 was obtained from Affymetrix (Santa Clara, California, USA). The TRIzol® Plus RNA Purification System (Gaithersburg, MD, USA) was used for further clean-up and concentrate RNA samples. BD Vacutainer® CPT™ (Cell Preparation Tube, Becton Dickinson (Cat # 362753, Franklin Lakes, NJ, USA) with Sodium Heparin was used for the separation of mononuclear cells from whole blood. PBS 1× sterilized solution was procured from Quality Biological Inc. (Gaithersburg, MD, USA).

2.2 Selection of human subject participants for in vitro studies

The study was undertaken with the prior approval by the Howard University Institutional Review Board (IRB-07-GSAS-30). The informed consent was obtained from volunteers attending Howard University. The subjects were 6 young adults (age range 17–20), 3 males and 3 females, who were interviewed about themselves and about their parents, prior to collection of blood. Subjects selected for this study were free from any major reported illness. They had not undergone any major surgical procedures that required general anesthesia during the last 5 years. Those with any major environmental exposure issues in their life time were excluded.

2.3 Human subjects for small population TLDA validation

Subjects for TLDA validation study primarily belong to a well-defined cohort of mother-and-children pairs, originally enrolled in the 'Slovak PCB Effects on Early Child Development Study' between 2001 and 2004 (Hertz-Picciotto et al., 2003, Sonneborn et al., 2008). Details of the recruitment and characterization of this cohort have been described elsewhere (Park et al., 2010). These children live in the Michalovce area, highly contaminated by PCB from a chemical manufacturing plant. We used the subjects (n=71; Male=30 Nos., Female=41 Nos.) solely based on their PCBs concentration (with the average blood PCB 3.02 ± 1.3 ng/mg of serum lipid; >75 percentile) from our earlier studies (Dutta et al., 2012, Mitra et al., 2012); they were included in this study only if they were free any clinical symptoms of chronic disease. Subject with no/background exposure level served as control. The Slovak Population RNAs were prepared from the whole blood and processed as previously described (Ghosh et al., 2013).

2.4 In Vitro exposure

The collected tubes of blood were brought into the lab immediately for isolation of PBMC cells. The detailed procedure can be found in our previous publications (Ghosh et al., 2011). The top five PCB congeners (PCB-118, PCB 138, PCB 153, PCB 170, and PCB 180) were the chemicals of our interest due to its maximum prevalence in the Slovak human exposed population. We chose 0.08 ng/ml of PCB 118, 0.87 ng/ml of PCB 138, 1.38 ng/ml of PCB 153, 0.52 ng/ml of PCB 170, and 1.24 ng/ml of PCB 180 according to the median concentration of PCBs (of human equivalence) in our exposed Slovak population (Hertz-Picciotto et al., 2003, Sonneborn et al., 2008). These PCBs (dissolved in DMSO) were added to each plate individually (in triplicate for each subjects), where the final concentration of DMSO was 0.1%. The cells remained exposed for 48 h. Control cell lines (vehicle control) were allowed to grow with DMSO only (0.1% of the total medium v/v) to ensure that the changes seen were not due to DMSO.

2.5 RNA preparation

RNA was extracted from the PBMC cells using a TRIzol® Plus RNA Purification System according to manufacturer's direction. Prior to isolation, the cells from each plates were washed twice with $1 \times$ PBS. The RNAs were re-solubilized in RNase-free water. Contaminating DNA was removed with the Ambion DNA-free kit. RNA concentrations were determined spectrophotometrically on a nanodrop at 230, 260 and 280 nm. RNA quality was also verified by Agilent bioanalyzer analysis using a RNA 6000 nanochip before microarray chip hybridization. The RNA was stored at -80°C .

2.6 cDNA Synthesis

Total RNA was reverse-transcribed to cDNA by using High-Capacity cDNA Reverse Transcription Kits (Part # 4387406; Applied Biosystems, CA, USA) according to manufacturers instruction. The reaction mixture (20 µL total volumes) was incubated at 25°C for 10 min and then at 37°C for 60 min followed by 95°C for 5 min. Finally, the mixture was heated at 95°C for 5 min. The cDNAs were stored in -15 to -25°C , if not used immediately (within 24 hours), or stored in 2 to 8°C .

2.7 Microarrays

The RNAs were reversely transcribed to cDNA with an oligo-dT primer containing T7 RNA polymerase promoter. The cDNA was 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 prior to hybridizing to Affymetrix Human Genome Array for 16 h (in triplicate for each subjects). 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 for quality control. The significant gene list identified with GeneSpring and dChip with Affymetrix probe set ID were imported into the dChip and clustered based on similarity in expression. Human Genome U133 Plus 2.0 Array in our microarray gene expression analysis for PCB exposure studies includes 54,675 probe sets, and was used during this study. The scan report generated by Gene Chip Operating Software (GCOS) had a scaling factor between 0.5 and 5, total percent of 'P-calls' between 30% and 50%, external controls cre>BioD>BioC>BioB and internal control was 1.0 ± 0.1 (pivot table). This pivot table was then further evaluated by Hierarchical Clustering Explorer (HCE) (Seo and Shneiderman, 2002). The clustering of HCE that has been done by row by row normalization (mean $\pm$ SD) and Euclidian distance was calculated with average linkage that shows unique clustering of test and control subjects together while analyzing our data (Figure 1). After this final quality control, the data were analyzed with Partek® Genomics Suite™.

2.8 Gene expression data analysis

The microarray data (*.cel file) were filtered and normalized by PLIER (http://www.affymetrix.com/support/technical/technotes/plier_technote.pdf) (Seo and Hoffman, 2006), and a subsequent statistical analysis was performed using the Partek® Genomics Suite™ analysis tool. Differential expression was compared using unpaired t-test statistics. A hierarchical clustering algorithm using the Pearson correlation was then used to temporally group those probe sets based on their expression pattern. Differential expression was determined by Partek's Paired t-test and filtered with p-value <0.05. Gene's annotations were expanded and upgraded using NCBI Entrez Gene, Unigene, and PubMed ID for all significantly different genes. "Minimum Information about a Microarray Experiment" (MIAME) compliant data has been submitted to the Gene Expression Omnibus (GEO) database. The datasets used in this paper can be accessed from the following GEO links: GSE22668.

To find common genes between the two data sets, i.e., *in vitro* (the current experimental) and our previous population studies (GSE22868, Dutta et al., 2012), the differentially expressed genes were compared by Partek Genomic Suites™ (version 6.6). We found 13 genes that are common in these two datasets (t-test, with FDR of $p < 0.05$). Those are APC, ARNT, CD3G, CYP1A1, CYP2D6, ENTPD3, ITGB1, LEPR, LRP12, MYC, RRAD, TAB1, and TRAP1. We chose TP53 gene under our current investigation as a major molecule as revealed through IPA analysis (Fig. 3) which activates several cancer signaling pathways in our experimental study, resembling the human studies that provided prior

suggestive evidence that PCBs are carcinogenic (ATSDR, 2000, Faroon et al., 2001, Laden et al., 2002).

2.9 Analysis towards the identification of cellular processes and pathways involved by Ingenuity Pathway Analysis (IPA)

Data sets containing gene identifiers and corresponding expression values (fold change) were uploaded into Ingenuity Pathway Analysis software (Ingenuity® Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base. We utilized the information in the Ingenuity Knowledge Base (Genes Only) as a reference set that consider both direct and indirect relationships. We included the molecules and/or the relationships only. To enrich our pathway analysis, we incorporated additional 661 gene transcripts from IPA knowledge base to our results. We used the data sources from ingenuity expert findings and use the “Core Analysis” function to interpret the data in the context of biological processes, pathways and networks. Differentially expressed gene identifiers were defined as value parameters for analysis and identified the relationship between gene expression alterations and related changes in biofunctions under the subcategories of Molecular and Cellular Functions, Physiological System Development and Function, and Disease and Disorders. Genes differentially expressed with $p < 0.05$ were overlaid onto global molecular networks developed from information contained in the knowledge base. Networks were then algorithmically generated based on their connectivity. Networks were “named” on the most prevalent functional group(s) present. Canonical Pathway (CP) analysis identified function specific genes significantly present within the networks.

2.10 High-throughput Taqman® low density array (TLDA)

To identify altered gene expression, we used predesigned TLDA cards (Applied Biosystems®, CA) to examine the expression of 14 common genes as identified in the methods described above (including IPA knowledge base) in the experimental subjects (*in vitro*), and 6 genes of interest (out of 14, based on our prior investigations and epidemiological disease manifestation in PCBs-exposed population) to examine in our exposed Slovak population through a small population validation study.

For the present study, the TaqMan Low-density Array card was configured into a 14 genes set (triplicate per assay within the TLDA card, see Supplemental table-1 for detector probe information). Each set of genes contained two endogenous control genes, GAPDH and 18s RNA. The RNAs were synthesized with the cDNA (5 μ L) and was mixed with 45 μ L of H₂O and 50 μ L of 2× TaqMan Universal PCR Mix. Each sample (100 μ L) was loaded into a port of the micro-fluid card, centrifuged, and run on an ABI Fast 7900HT System (ABI, CA) for 2 min at 50 °C, 10 min at 94.5 °C, followed by 40 cycles for 30 sec. at 97 °C and 1 min at 59.7 °C.

2.11 TLDA data analysis

The TLDA data were analyzed by SDS Ver. 2.4 software (ABI, CA). Threshold cycle (Ct) data for all target genes and control gene 18s RNA were used to calculate Ct values [Ct = Ct (target gene) - Ct (18s RNA)]. Then, Ct values were calculated by subtracting the

calibrator (control) from the Ct values of each target. To visualize and further expression analysis, the data were exported in plate centric format to DataAssit V2.0 (ABI, CA) which allowed us to inspect the status of a gene(s) in the respective groups of lower and higher PCB exposures.

3. Results

3.1 Differential expression of genes with mixed PCBs exposure in vitro

Under the experimental conditions, we present here the top 100 genes that were differentially expressed (both up/down-regulated with 1.5 fold change, and statistically significant t-test, with FDR of $p < 0.05$) (Fig. 1): 16% were up-regulated and 84% were down-regulated, when compared to control (Table 1).

3.2 The effects of the differentially expressed genes

The list of biological effects caused by exposure to mixed PCBs can be found in three levels; gene function level (Table 1), network level (Table 2), and Bio-functions level (Table 3). Analysis of the genes identified above revealed six significant genetic networks (score 10.0), as listed in Table 2. The top-scoring networks include: *Endocrine System Disorders*, *Genetic Disorders*, *Metabolic Diseases*, *Cancer*, *Auditory Diseases*, *Dermatological Disease and Conditions*, *Developmental disorders*, *Hematological Disease*, *Immunological Disease*, and *Neurological Disease* (Figure 2; panel B). Among the genes in the Molecular and Cellular Functions network, *Cellular Assembly & Organization*, *Carbohydrate Metabolism*, *Lipid Metabolism*, *Small Molecule Biochemistry*, and *Cell Cycle* functions were revealed (Figure 2; panel C). In the Physiological System Development and Functions network, notable functions were *Lymphoid Tissue Structure & Development*, *Embryonic Development*, *Hematological System Development & Function*, *Tissue Development*, *Cardiovascular system Development & Function*, *Tissue Morphology and Tumor Morphology* (Figure 2, panel A).

3.3 The canonical pathways and GO enrichment of biological processes in vitro

In the canonical pathway analysis, we chose to build the pathways connecting the top 3 networks (Networks 1–3, Score 30, Table 3). The top thirteen (13) pathways identified through this approach were: *Insulin Receptor Signaling*, *Apoptosis Signaling*, *Aryl Hydrocarbon Receptor Signaling*, *p53 Signaling*, *G-protein Coupled Receptor Signaling*, *Ovarian Cancer Signaling*, *Prostate Cancer Signaling*, *Molecular Mechanisms of Cancers*, *Chronic Myeloid Leukemia Signaling*, *Type I and Type II Diabetes Mellitus Signaling*, *Cardiac Hypertrophy Signaling*, and *Colorectal Cancer Metastasis Signaling* (Fig. 3). Further in-depth analysis also identified some important pathways, viz., *Leptin Signaling in Obesity*, *Endometrial Cancer Signaling*, *Cell-cycle: G2/M DNA Damage Checkpoint Regulation*, *NF- κ B Signaling*, some of which are in accord with our previous investigations (Ghosh et al., 2010, 2011, 2013, 2014).

3.4 Top biofunctions and Disease & Disorder development in in vitro

The Gene Set Enrichment Analysis (GO Enrichment Score) revealed altered expression of genes with important and shared common biological functions, chromosomal location, or

regulation, e.g. *Developmental process, Biological Recognition, Biological Adhesion, Reproduction, Death, Cellular Compartment Organization, Pigmentation, and Reproductive Process*. Furthermore, the IPA data analysis revealed 17 important biofunctions in the diseases and disorder categories of Cancer, Genetic Disorders, Reproductive System Disease, Skeletal and Muscular Disorder, Infection Mechanism, Renal and Urological Disease, Dermatological Disease, Connective Tissue Disorders, Endocrine System Disorders, Gastrointestinal Disease, Neurological Disease, Developmental Disorder, Hematological Disease, Immunological Disease, Cardiovascular Disease, and Inflammatory Response (Fig. 4).

3.5 In vitro and Population Validation of Selected Genes through TLDA

High throughput quantitative real time PCR (TLDA in ABI platform) confirmed the PCBs-associated altered expression of our 14 common genes under the *in vitro* experimental condition. All 14 genes were well amplified in our experimental condition (cross-validation in mixed-PCBs *in vitro* exposures, n=6; Table-4, Fig. 5 & 6).

Some of the signature genes, *CYP2D6, LEPR, LRP12, ARNT, RRAD, and TP53*, which we presume could serve as putative biomarkers under such an exposure scenario, were further cross-validated in the PCBs-exposed human population for their consistency (Fig. 6, panel A–F). The genes *LEPR, LRP12, ARNT*, and *TP53* were well confirmed in the population samples (Figure 6, panel B, C, D, and F respectively). The *CYP2D6* and *RRAD* genes were either up-regulated in the experiments or down-regulated in the population, or vice-versa (Fig. 6, panel A & E respectively).

4. Discussion

In the field of toxicology, most of the studied agents, including PCBs, are likely to exert their adverse effects directly or indirectly by altering “normal” signaling processes in the cell. Moreover, adverse end points resulting from toxicant exposures may be profiled and compared to normal tissue/cell samples to discern differences in gene expression between the two states (Hamadeh et al., 2002, Waring et al., 2001). Our overall hypothesis was based on several epidemiological studies showing that PCBs can cause a wide range of health effects: we posited that those health effects were initiated by receptor binding/activation, leading to altered gene and protein expression. Under the present investigation, we observed that gene expression changes resulting from the exposure may indeed reflect an investigational approach that highlights biomarkers of specific diseases and disorders, e.g. metabolic disorders (including obesity and diabetes) cardiovascular, endocrine disruption, and cancers, in accord with results observed in epidemiological settings (Langer et al, 2014; Arrebola et al., 2014; Recio-Vega et al., 2013; Lee et al., 2014; Wadzinski et al., 2014; Pereira-Fernandes et al., 2014; Sexton et al., 2013, Casas et al., 2014). Research utilizing PBMC cells in human are now being widely used to test for the efficacy of new drugs and treatments, to divulge the differential gene expression patterns induced by toxicity, and to study downstream effectors, all of which can be used as a potential source for developing disease-specific biomarkers (Reynes et al., 2014; Saidijam et al., 2014; Roccaet et al., 2014; Kong 2014). In such a model, studying the gene expression changes might provide us a

valuable insights and better understanding of their mechanism(s) of action by highlighting which enzymes or proteins are targeted by these exposures.

The microarray analysis of data from this study clearly indicates that PCBs exposure caused significant differential gene expression (either up or down-regulated). We identified 14 genes for further study, viz. *APC*, *ARNT*, *CD3G*, *CYP1A2*, *CYP2D6*, *ENTPD3*, *ITGB1*, *LEPR*, *LRP12*, *MYC*, *RRAD*, *TAB1*, *p53*, and *TRAP1*, which showed notable expression changes and are known to have major impacts in mediating toxicities by altering cellular and molecular functions in developing disease and disorders under experimental condition, among which six were well validated in the PCBs-exposed Slovak population.

In the present *in vitro* study, *RRAD* was up-regulated in two of the exposed subjects in the PBMC study, and it was up-regulated in most of the subjects in the population validation study. *RRAD* over-expression is associated with insulin resistance in Type II (non-insulin-dependent) diabetes mellitus (Reynet and Khan, 1993). Rad (Ras associated with diabetes) GTPase is the prototypic member of a subfamily of Ras-related small G proteins, normally expressed in heart, skeletal muscle, and lung. Rad is over-expressed in skeletal muscle of some patients with type II diabetes mellitus and/or obesity. Over-expression of Rad inhibits glucose uptake in cultured muscle and fat cells (Moyers et al., 1996) and in adipocytes and muscle cells in culture, it results in diminished insulin-stimulated glucose uptake (Ilany et al., 2006). Our IPA core analysis of significant canonical pathways also highlighted the Type II diabetes mellitus signaling pathway and insulin receptor signaling.

The *ARNT* gene encodes the aryl hydrocarbon receptor nuclear translocator protein that forms a complex with ligand-bound aryl hydrocarbon receptor (AhR) (Reyes et al., 1992) and is required for receptor function and is involved in the induction of several enzymes that participate in xenobiotic metabolism. Induction of enzymes involved in xenobiotic metabolism occurs through binding of the ligand-bound AhR to xenobiotic responsive elements in the promoters of genes for these enzymes (Whitelaw et al., 1993). Non-ortho PCBs, also known as the coplanar PCBs, bind the AhR and are capable of producing dioxin-like effects within biological systems (Mortensen and Arukwe, 2008). In our *in vitro* (Fig. 5) and population validation studies (Fig. 6D), the AhR/*ARNT* gene was down-regulated during our TLDA validation that corroborates our 45 month gene expression studies earlier (Dutta et al., 2012). This AhR receptor signaling pathway was also important among our major canonical pathways through IPA analysis (Fig. 4).

Cytochrome P450 2D6 (*CYP2D6*), a member of the cytochrome P450 mixed-function oxidase system encodes a member of the cytochrome P450 superfamily of enzymes. While *CYP2D6* is involved in the oxidation of a wide range of substrates, there is considerable interest in its expression induced by xenobiotic materials, e.g., PCBs (Tabb et al., 2004) which increased our interest because of the reported poor neurobehavioral development in case of PCB-exposed children (Schantz, 1996, Stewart et al., 2008). The results on *CYP2D6* in our experimental condition showed that it was down-regulated in all the subjects in the PBMC experiment, and in most of those in the validation population (Fig. 6, panel A).

Leptin receptor also known as LEP-R is a protein that in humans is encoded by the *LEPR* gene. LEP-R functions as a receptor for the fat cell-specific hormone leptin that regulates body weight and is involved in the regulation of fat metabolism, as well as in a novel hematopoietic pathway that is required for normal lymphopoiesis (Bennett et al., 1996). In our experimental studies, the *LEPR* gene was down-regulated in all of the *in vitro* samples (Fig. 5), which is in accord with the status of this gene in a previous population study (Ghosh et al., 2013) that perfectly corroborated with the population validation analysis we report in the present paper (Fig. 6, panel B). Increasing evidence suggests that the commonly held causes of obesity, which are over-eating, inactivity and genetic pre-disposition, do not fully explain the current obesity epidemic. Interestingly, the production and use of synthetic chemicals have increased dramatically, in parallel with growing obesity and it has been suggested that EDCs may play a key role in obesity development by altering physiological control mechanisms (Tang-Péronard et al., 2011, Grün and Blumberg, 2009, Newbold et al., 2009, Elobeid et al., 2010). Among them, POPs, OC pesticides, and PCBs may be particularly interesting because low dose OC pesticides or PCBs were strongly linked to type 2 diabetes, insulin resistance, and metabolic syndrome, in all of which, obesity is believed to play a critical role (Lee et al., 2011, 2012, 2014).

Several pathways important in the molecular mechanisms of cancer were also identified in this study. In a recent study designed to evaluate the relation between PCB exposure and breast cancer risk in Mexican women, an association between heavy and potentially estrogenic PCB congeners and breast cancer risk was shown (Recio-Vega et al., 2011) corroborating our observations. Furthermore, based on epidemiological associations of PCBs and cancers at several organ sites, particularly the liver, biliary tract, intestines, and skin (melanoma), the human studies provide suggestive evidence that PCBs are carcinogenic (ATSDR, 2000, Faroon et al., 2001, Laden et al., 2002, Lauby-Secretan et al., 2013). Even after categorization of PCBs as cancer causing agents by ATSRD in 2002, the debate on the risk of cancer due to PCBs exposure in human is, however, highly controversial (Golden and Kimbrough, 2009) and has yet to be resolved.

In our experiments we observed that several important cancer-related genes were affected. *p53* (TP53) is crucial in multicellular organisms, where it regulates the cell cycle and, thus, functions as a tumor suppressor that is involved in preventing cancer, whereas the *c-MYC* protein participates in energy-consuming processes such as cell proliferation, ribosomal biosynthesis, glycolysis, mitochondrial functions, and differentiation and its expression is often dysregulated by human cancers (Dang, 1999). Specifically, we observed that *p53* was deregulated (down-regulated) in the experimental model and had a similar pattern of down-regulation in the majority of the subjects (Fig 6, panel F). Through a prior investigation, we also found that *p53* was down-regulated over a short exposure period, showing loss of cell viability and apoptosis, but was up-regulated over a chronic exposure of 12 weeks period (Ghosh et al., 2007), which corroborates our present observation concerning the involvement of cell cycle and cell death pathways in the molecular and cellular functions (Fig. 2C).

LRP12 gene may also be characterized by its differential expression in cancer cells. The product of this gene is predicted to be a transmembrane protein, and was found to be lower

in tumor derived cell lines compared to normal cells. LRP12 belongs to the LDLR superfamily and may play a role in signal transduction (Qing et al., 1999; Battle et al., 2003). Information on the role of LRP12 is scanty, especially while considering its involvement in some environmental toxic exposures. The work by Garnis et al., (2004) suggested that LRP12 might function as an oncogene in oral tumors. To date, we may be the first to report that this LRP12 gene may be related to PCBs toxicity.

Regarding our findings of pathways involving Type 2 diabetes, Obesity, Cardiovascular diseases, and even cancer, there is considerable evidence that the risks of these diseases may begin early in life, during pregnancy, and early childhood. There are numerous studies showing that rapid weight gain in the first few months of life is associated with obesity later in life (Boekelheide et al., 2012). Another recent study also suggests that other prenatal OC concentrations (PCBs, DDE, and DDT) were associated with being overweight at 6.5 years of age (Valvi et al., 2012). Obesity like other complex diseases is caused by myriad interactions between genetic, behavioral and environmental factors. There is an emerging hypothesis, based on data from several chemicals in animal studies, that the obesity epidemic could be due to chemical exposures during vulnerable windows of development, mainly *in utero* and the first few years of life (Lubrano et al., 2013; Heindel JJ, vom Saal 2009), as previously observed in our Slovak epidemiological investigations. Metabolic syndrome is also associated with the rise in obesity and may progress to type 2 diabetes. There is significant data supporting the idea that metabolic syndrome is programmed during development and that there is a role for maternal diet in its etiology (Boekelheide et al, 2012). While there are few data linking developmental exposures to environmental chemicals to actual metabolic syndrome, there are data showing effects of exposures on the development of obesity and type 2 diabetes (Uemura, 2012; Faerch et al., 2012, Ghosh et al., 2014).

During last decade, after completion of human genome project, the biomedical research community is focusing increased attention on finding biological markers (Biomarkers) so that early diagnosis and even prevention can be achieved, before the symptoms appear (Thayer et al., 2012, Sexton and Salinas 2014, Ghosh et al., 2014).

Therefore, developing a rich set of biomarkers for monitoring early health effects in the life course is becoming more important. Biomarker-based methods capable of identifying high-risk individuals with specificity and selectivity will greatly facilitate early detection directed towards reducing environmentally induced diseases, such diabetes, obesity, hearing impairments, and malignancies, as they have already started appearing in PCB-exposed population. Such efforts are also shedding new light on possible mechanisms for the genesis of disease development.

5. Conclusion

The results from the present study provide an integrated view of gene expression and potential downstream pathophysiological changes that might lead PCB-exposed subjects towards the development of diseases and disorders. The results thus provide a possibility to develop a screening method using these gene fingerprints that could lead to the identification

of subgroups at high risk, well ahead of time, even before the actual disease becomes visible. If validated through population-based studies, such a comprehensive approach will generate new information and fill critical gaps in knowledge regarding PCB exposure-related human health effects and potentially open the door for the development of early preventive strategies.

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Novelty

- Knowledge of disease pathways that were associated with human equivalence PCB exposures.
- Identified discrete gene sets that were perturbed in the *in vitro* experiments.
- Evaluated and validated the candidate genes status by comparison to the population study.
- Pathways for Obesity, Type 2 diabetes, and Cancers towards potential disease development.
- Explored the potential use of these gene fingerprints as disease susceptibility.

Fig. 1. Hierarchical cluster analysis along with the heat map of the differentially expressed gene set (100) in human PBMCs *in vitro* study following human equivalence PCBs exposures. Red denotes up-regulation, blue down-regulation, and gray no difference; where brighter color (+/-) denotes the increasing intensities of up/down-regulations induced by PCBs exposures. The Hierarchical clustering (Dendrogram) displayed the results systematically, and showed that control and treated are grouped together and was based on average linkage with Pearson correlation.

Fig. 2. The key (Top) bio-functions in developing toxicities with the differentially expressed gene set following PCBs mixed-exposures in vitro as obtained through IPA analysis physiological system development and functions (**A**), disease and disorder development (**B**), and in molecular and cellular functions (**C**). The most statistically significant top biofunctions that were identified in the IPA Tox analysis are listed here according to their p value ($-\text{Log}$). The threshold line corresponds to a p value of 0.05.

Fig. 3.

Connectivity of differentially expressed genes in the important signaling pathway following mixed PCBs exposure in human PBMC *in vitro* depicting the connectivity between genes expressed (with 1.5 fold change, t-test, $p < 0.05$). Geometric figures in red denote up-regulated genes and those are green indicate down-regulation. Genes in the top 6 networks (our experimental 100 gene sets) were allowed to grow our pathway with the direct/indirect relationship from the IPA knowledge base with the stringent filter, experimentally observed, those who were only from human study. Solid interconnecting lines show the genes that are directly connected and the dotted lines signify the indirect connection between the genes and cellular functions. Canonical functions (signaling) that are highly represented are shown within the box. Genes in uncolored notes were not identified as differentially expressed in our experiment and were integrated into computational generated networks based on evidence stored in the *IPA* knowledge base indicating relevance of this network.

Fig. 4.

Top biofunctions in disease and disorder development with the *in vitro* studies (PCBs mixed) generated through IPA analysis. The gene sets from the study were filtered, uploaded, and run through in the IPA comparative data Analysis module. The important disease and disorders that are represented here were at or above the threshold value (corresponds to a p value of 0.05). Fischer's exact test was used to calculate a p -value determining the probability that each biological function and/or disease assigned to the dataset.

Fig. 5.

In vitro Quantitative Real-time PCR (qRT-PCR) validation of the selected 14 genes (both experimental and IPA knowledge base) by Taqman Low Density Array (TLDA) in ABI platform (7900HT Fast Real-Time PCR System) after analyzed by SDS RQ Manager Version 1.2.1 ($\Delta\Delta C_t$). Each panel shows the relative quantification of the selected genes up/down-regulation among the experimentally exposed condition (Subjects 1–6). The relative quantification is calculated in contrast to calibrator samples, i.e.; no-exposure in *in vitro* studies (control).

Fig. 6. Quantitative Real-time PCR (qRT-PCR) validation of the selected 6 genes of interest by Taqman Low Density Array (TLDA) in ABI platform (7900HT Fast Real-Time PCR System) after analyzed by SDS RQ Manager Version 1.2.1 ($\Delta\Delta C_t$). The panels A–F (with the respective genes) represent the relative quantification of the genes upon small population validation (the population with high PCBs in their blood; $n=71$) with the same gene transcript that has been used in *in vitro* studies. The relative quantification is calculated in contrast to calibrator samples, i.e., the subjects with no/background PCBs exposures in the population.

Table 1

List of 100 annotated gene transcription that differentially expressed in human PBMC (1.5 fold change, t-test, $p < 0.05$) following exposure to human equivalence (of Slovak population) mixed PCBs.

Probeset ID	Gene Symbol	Gene Title	Fold-Change
1555118_at	ENTPD3	Ectonucleoside triphosphate diphosphohydrolase 3	-3.29083
1554726_at	ZNF655	Zinc finger protein 655	-1.5349
1555762_s_at	RBM15	RNA binding motif protein 15	-1.72036
1555837_s_at	POLR2B	Polymerase (RNA) II (DNA directed) polypeptide B, 140kDa	-1.66457
1556006_s_at	CSNK1A1	Casein kinase 1, alpha 1	-1.60048
1558688_at	LOC441461	Hypothetical-LOC441461	1.51083
201177_s_at	UBA2	Ubiquitin-like modifier activating enzyme 2	-1.6631
201243_s_at	ATP1B1	ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	-2.10046
201437_s_at	EIF4E	Eukaryotic translation initiation factor 4E	-1.77381
201449_at	TIA1	TIA1 cytotoxic granule-associated RNA binding protein	-1.97483
201450_s_at	TIA1	TIA1 cytotoxic granule-associated RNA binding protein	-1.74723
201523_x_at	UBE2N	Ubiquitin-conjugating enzyme E2N (UBC13 homolog, yeast)	-1.58113
202318_s_at	SENP6	SUMO1/sentrin specific peptidase 6	-1.83591
202320_at	GTF3C1	General transcription factor IIIC, polypeptide 1, alpha 220kDa	1.546298
202487_s_at	H2AFV	H2A histone family, member V	-1.55526
202653_s_at	7-Mar	Membrane-associated ring finger (C3HC4) 7	-1.95386
203011_at	IMPA1	Inositol(myo)-1(or 4)-monophosphatase 1	-2.13757
203017_s_at	SSX2IP	Synovial sarcoma, X breakpoint 2 interacting protein	-2.11359
203405_at	PSMG1	Proteasome (prosome, macropain) assembly chaperone 1	-1.81846
203552_at	MAP4K5	Mitogen-activated protein kinase kinase kinase kinase 5	-1.71411
203855_at	WDR47	WD repeat domain 47	-1.6205
204299_at	SFRS13A	Splicing factor, arginine/serine-rich 13A	-1.83462
206804_at	CD3G	CD3g molecule, gamma (CD3-TCR complex)	1.65203
208805_at	PSMA6	Proteasome (prosome, macropain) subunit, alpha type, 6	-1.9818
208808_s_at	HMGB2	High-mobility group box 2	-1.84785
209096_at	UBE2V2	Ubiquitin-conjugating enzyme E2 variant 2	-1.51946
209422_at	PHF20	PHD finger protein 20	-1.50916
209666_s_at	CHUK	Conserved helix-loop-helix ubiquitous kinase	-1.50891
210285_x_at	WTAP	Wilms tumor 1 associated protein	-1.70726
211354_s_at	LEPR	Leptin receptor	-2.10571
211967_at	TMEM123	Transmembrane protein 123	-2.29464
212281_s_at	TMEM97	Transmembrane protein 97	-2.06445
212426_s_at	YWHAQ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, theta po	-1.63874
212536_at	ATP11B	ATPase, class VI, type 11B	-1.581
212557_at	ZNF451	Zinc finger protein 451	-1.50106
212824_at	FUBP3	Far upstream element (FUSE) binding protein 3	-1.62965
212880_at	WDR7	WD repeat domain 7	-1.83247
213225_at	PPM1B	Protein phosphatase, Mg ²⁺ /Mn ²⁺ dependent, 1B	-1.65843

Probeset ID	Gene Symbol	Gene Title	Fold-Change
213227_at	PGRMC2	Progesterone receptor membrane component 2	-1.59415
213262_at	SACS	Spastic ataxia of Charlevoix-Saguenay (sacsin)	-2.30661
214429_at	MTMR6	Myotubularin related protein 6	-2.26117
215684_s_at	ASCC2	Activating signal cointegrator 1 complex subunit 2	2.37375
215716_s_at	ATP2B1	ATPase, Ca++ transporting, plasma membrane 1	-1.75211
215783_s_at	ALPL	Alkaline phosphatase, liver/bone/kidney	2.62704
216060_s_at	DAAM1	Dishevelled associated activator of morphogenesis 1	-1.5949
216680_s_at	EPHB4	EPH receptor B4	1.64141
216755_at	OSBPL10	Oxysterol binding protein-like 10	1.52037
217203_at	GLULP4	Glutamate-ammonia ligase (glutamine synthetase) pseudogene 4	1.55534
217745_s_at	NAA50	N(alpha)-acetyltransferase 50, NatE catalytic subunit	-1.62715
217945_at	BTBD1	BTB (POZ) domain containing 1	-1.59839
218171_at	VPS4B	Vacuolar protein sorting 4 homolog B (S. cerevisiae)	-1.63559
218352_at	RCBTB1	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing prot	-1.66994
218396_at	VPS13C	Vacuolar protein sorting 13 homolog C (S. cerevisiae)	-1.66178
218846_at	MED23	Mediator complex subunit 23	-1.7511
219630_at	PDZK1IP1	PDZK1 interacting protein 1	2.32042
219811_at	DGCR8	DiGeorge syndrome critical region gene 8	1.78871
220175_s_at	CBWD1-D7	COBW domain containing 1 -7	-1.54632
220253_s_at	LRP12	Low density lipoprotein receptor-related protein 12	-2.10571
222292_at	CD40	CD40 molecule, TNF receptor superfamily member 5	1.54035
222555_s_at	MRPL44	Mitochondrial ribosomal protein L44	-1.54162
222805_at	MANEA	mannosidase, endo-alpha	-2.24407
222825_at	OTUD6B	OTU domain containing 6B	-1.59575
222924_at	SLMAP	Sarcolemma associated protein	-1.60831
223288_at	USP38	Ubiquitin specific peptidase 38	-1.98296
223444_at	SENp7	SUMO1/sentrin specific peptidase 7	-1.82933
223875_s_at	EPC1	Enhancer of polycomb homolog 1 (Drosophila)	-1.5408
224281_s_at	NGRN	Neugrin, neurite outgrowth associated	-1.69305
224691_at	UHMK1	U2AF homology motif (UHM) kinase 1	-1.9501
224720_at	MIB1	Mindbomb homolog 1 (Drosophila)	-3.03948
224725_at	MIB1	Mindbomb homolog 1 (Drosophila)	-2.71137
224928_at	SETD7	SET domain containing (lysine methyltransferase)	-1.56236
225213_at	PPTC7	PTC7 protein phosphatase homolog (S. cerevisiae)	-1.59082
225290_at	ETNK1	Ethanolamine kinase 1	-2.54633
225351_at	FAM45A	Family with sequence similarity 45, member A	-1.6578
225367_at	PGM2	Phosphoglucomutase 2	-1.80992
225772_s_at	C12orf62	chromosome 12 open reading frame 62	-1.63556
225892_at	IREB2	Iron-responsive element binding protein 2	-1.82595
226109_at	C21orf91	Chromosome 21 open reading frame 91	-1.94281
226140_s_at	OTUD1	OTU domain containing 1	-2.09994
226284_at	ZBTB2	Zinc finger and BTB domain containing 2	-1.58058

Probeset ID	Gene Symbol	Gene Title	Fold-Change
226432_at	ETNK1	Ethanolamine kinase 1	-2.68345
226520_at	LCOR	Ligand dependent nuclear receptor corepressor	-2.03599
226667_x_at	EPN1	Epsin 1	1.63073
226921_at	UBR1	Ubiquitin protein ligase E3 component n-recognin 1	-2.25037
227003_at	RAB28	RAB28, member RAS oncogene family	-1.94344
227239_at	FAM126A	Family with sequence similarity 126, member A	-1.6925
227375_at	ANKRD13C	Ankyrin repeat domain 13C	-1.74244
227708_at	EEF1A1	Eukaryotic translation elongation factor 1 alpha 1	-1.89046
227757_at	CUL4A	Cullin 4A	2.22597
227787_s_at	MED30	Mediator complex subunit 30	-1.5697
228312_at	PI16	Peptidase inhibitor 16	1.57939
228392_at	ZNF302	Zinc finger protein 302	-1.83879
228751_at	CLK4	CDC-like kinase 4	-2.00776
228941_at	ALG10B	Asparagine-linked glycosylation 10, alpha-1,2-glucosyltransferase homolog B	-2.07412
230029_x_at	UBR3	Ubiquitin protein ligase E3 component n-recognin 3 (putative)	-1.94883
233184_at	EPHA6	EPH receptor A6	1.87456
235396_at	C22orf25	Chromosome 22 open reading frame 25	1.51629
239482_x_at	ZNF708	Zinc finger protein 708	-1.84726
240941_at	ITSN2	Intersectin 2	1.7006
242293_at	ING3	Inhibitor of growth family, member 3	-1.54031

Table 2

Significant Top Bio-function from IPA knowledge base associated with differentially expressed genes in mixed-PCB exposure *in vitro*.

Category	Important Molecule(s)/(Genes)	...log (p-value)
Disease & Disorders		
<i>Endocrine System Disorders</i>	<i>LEPR, UBR1</i>	2.42E-03-5.72E-03
<i>Genetic Disorder</i>	CD40, LEPR, DGCR8, UBR1, RBM15, ALPL	2.42E-03-2.83E-02
<i>Metabolic Disease</i>	<i>LEPR, UBR1, ALPL</i>	2.42E-03-5.72E-03
<i>Cancer</i>	CD3G, EPN1, CD40, ITSN2, RBM15, CHUK, EIF4E	5.72E-03-3.84E-02
<i>Auditory Diseases</i>	<i>UBR1</i>	5.72E-03-5.72E-03
<i>Dermatological Diseases and Conditions</i>	<i>UBR1</i>	5.72E-03-5.72E-03
<i>Developmental Disorder</i>	DGCR8, RCBTB1, UBR1, ALPL	5.72E-03-1.14E-02
<i>Hematological Disease</i>	CD3G, CD40, RBM15, CHUK	5.72E-03-3.84E-02
<i>Immunological Disease</i>	CD3G, CD40, CHUK	
<i>Neurological Disease</i>	<i>UBR1, OSBPL10, EEF1A1, UBR3, NGRN, MTMR6, SLMAP, CD40, PPM1B, MIB1, C21orf91, ITSN2, SSX2IP, WDR7, HMGB2</i>	5.72E-03-3.56E-02
<i>Skeletal and Muscular Disorders</i>	<i>C21orf91, CD40, EEF1A1, HMGB2, ITSN2, MIB1, MTMR6, NGRN, OSBPL10, PPM1B, SLMAP, SSX2IP, UBR3, WDR7</i>	3.56E-02
Molecular and Cellular Functions		
<i>Cellular Assembly & Organization</i>	<i>EEF1A1, EIF4E, UBE2N, TMEM123, SACS</i>	1.13E-03-2.83E-02
<i>Carbohydrate Metabolism</i>	CD40, EEF1A1, ETNK1, IMPA1, MTMR6	2.13E-03-4.49E-02
<i>Lipid Metabolism</i>	CD40, EEF1A1, ETNK1, IMPA, ATP11B, MTMR6	2.13E-03-4.49E-02
<i>Small Molecule Biochemistry</i>	CD40, EEF1A1, ETNK1, IMPA, ATP11B, MTMR6	2.13E-03-4.49E-02
<i>Cell Cycle</i>	<i>CHUK, CSNK1A1, EIF4E, ZNF655, CUL4A, CD40, YWHAQ, UHMK1</i>	5.72E-03-4.22E-02
Physiological System Development and Function		
<i>Lymphoid Tissue Structure & Development</i>	CD40, CHUK	1.72E-03-4.49E-02
<i>Embryonic Development Hematological System</i>	<i>TMEM123, EPHB4, CUL4A, EIF4E</i>	5.72E-03-3.38E-02
<i>Development & Function</i>	CD40, CUL4A, EIF4E, CHUK	5.72E-03-4.49E-02
<i>Tissue Development</i>	<i>TMEM123, CD40, CHUK, EPHB4, LEPR, EIF4E</i>	5.72E-03-3.38E-02
<i>Cardiovascular system Development & Function</i>	EPHB4	1.14E-02-1.71E-02
<i>Tissue Morphology</i>	CD40, CUL4A, EIF4E, CHUK, LEPR	1.62E-02-3.38E-02
<i>Tumor Morphology</i>	CD40, CUL4A, CHUK	1.62E-02-2.83E-02

Genes in **Bold** = Up-regulated; *Italicized* = Down-regulated; p-value = Fischer's exact test was used to calculate a p-value determining the probability that each biological function and/or disease assigned to that dataset.

Table 3

High-scoring networks (Score >10) identified by Ingenuity® Pathway Analysis in PCB mixed exposure to Human PBMC. Top 6 (Six) out of 24 networks are represented here.

Network ID	Genes in Network	Score	Focus Molecules	Functions
1.	ASCC2 , <i>ATP1B1</i> , ATPase, Caspase, CD40 , <i>CHUK</i> , <i>CUL4A</i> , <i>EIF4E</i> , <i>EPC1</i> , <i>GTF3C1</i> , HISTONE, <i>ZNF451</i> Ikk (family), <i>ING3</i> , Interferon alpha, <i>LEPR</i> , <i>YWHAQ</i> , <i>LOC100505793/SRSF10</i> , <i>MAP4K5</i> , <i>MARCH7</i> , <i>MED23</i> , <i>MED30</i> , <i>MIB1</i> , IKK (complex), NFkB (complex), PI3K (complex), <i>POLR2B</i> , <i>PPM1B</i> , RNA polymerase II, TRAP/Media, <i>UBE2N</i> , <i>UBE2V2</i> , Vegf, <i>VPS4B</i> , <i>WTAP</i>	51	24	Gene Expression, Cell Death, Antigen Presentation
2.	<i>ALG10B</i> , <i>ATP11B</i> , <i>BTBD1</i> , CALB1, <i>CBWD1</i> , DCAF16, <i>ETNK1</i> , <i>IMPA1</i> , KCNN4, LARP1, <i>LCOR</i> , <i>MANEA</i> , <i>MIB1</i> , miR-344d/miR-410, miR-520d-5p/miR-524-5p, miR-590-3p, <i>MTMR6</i> , <i>MTMR9</i> , <i>PAPSS1</i> , <i>PPTC7</i> , <i>RAB28</i> , <i>RAP1GDS1</i> , <i>RNF182</i> , <i>RSL1D1</i> , <i>SAMSN1</i> , <i>SENP7</i> , <i>SETD7</i> , <i>SSX2IP</i> , <i>SUV39H2</i> , <i>UBR3</i> , <i>USP38</i> , <i>USP53</i> , <i>ZNF655</i> , <i>ZNF708</i> , <i>ZNRF1</i>	39	19	Carbohydrate Metabolism, Cell Morphology, Cellular Functions & Maintenance
3.	ACTN2, ASCC3 , <i>ATP11B</i> , <i>C21orf91</i> , <i>CLK4</i> , <i>SIKE1</i> , DGCR8 , DROSHA, <i>FAM40B</i> , <i>FUBP3</i> , H2AFV, <i>LRP12</i> , <i>MANEA</i> , miR-194, miR-514, miR-606, <i>MOBK13</i> , miR-219-2-3p/miR-219-3p, miR-297a/miR-297, <i>UBR3</i> , miR-548c-3p, miR-802 (human), <i>CTTNBP2NL</i> , <i>NAA16</i> , <i>NAA50</i> , <i>OTUD6B</i> , <i>PGM2</i> , <i>QKI</i> , <i>SENP6</i> , <i>SLMAP</i> , <i>TIA1</i> , <i>TIAL1</i> , <i>TMEM123</i> , <i>TOB1</i> , <i>UBE2B</i>	31	16	Cellular Growth & Proliferation, Lipid Metabolism, Molecular Transport
4.	<i>ANKRD13C</i> , <i>ATP1A2</i> , <i>DMXL2</i> , <i>DNAJB11</i> , DROSHA, <i>E2F1</i> , <i>EFNA3</i> , <i>EFNB2</i> , EPHB4 , <i>FAM126A</i> , <i>FAM45A</i> , <i>HSPE1</i> , ITSN2 , <i>MSL1</i> , <i>NGRN</i> , <i>NPLOC4</i> , OSBPL10 , <i>PAX9</i> , PI16 , <i>PTPN4</i> , <i>PTPRJ</i> , <i>RCBTB1</i> , <i>RNF144A</i> , <i>SACS</i> , <i>SRGAP2</i> , <i>SSX2IP</i> , <i>SUV420H1</i> , <i>TMEM123</i> , <i>WDR7</i> , <i>UBE2B</i> , <i>UBR1</i> , <i>VPS13C</i> , miR-205, miR-27b/miR-27a, miR-20a/miR-106b/miR-17-5p (includes others)	29	15	Cardiovascular System Development & Function, Organismal Development, Cell Morphology
5.	<i>ACO2</i> , <i>ADRM1</i> , <i>C12orf11</i> , <i>C12orf62</i> , <i>CDKN1B</i> , <i>CKS2</i> , <i>COPS7A</i> , <i>COPS7B</i> , <i>DAAMI</i> , DCAF8, EGFR, <i>ENTPD3</i> , EPN1 , <i>ETNK1</i> , <i>GRWD1</i> , <i>HBXIP</i> , <i>HNF4A</i> , <i>IKBKE</i> , <i>IREB2</i> , miR-1264, miR-16/miR-497/miR-195 (includes others), miR-802 (human), <i>MRPL44</i> , <i>NAA10</i> , <i>NAA20</i> , <i>NFKBIL1</i> , <i>PGRMC2</i> , <i>PHF20</i> , <i>PRPF38A</i> , <i>PSMA6</i> , <i>RBM15</i> , <i>SDHB</i> , <i>TGFB1</i> , <i>TIA1</i> , <i>UHMK1</i>	23	13	Cellular Growth & Proliferation, Cell Death, Cellular Function & Maintenance
6.	ALPL , <i>ANXA3</i> , <i>ATP2B1</i> , <i>BRF1</i> (includes EG:2972), <i>CCL18</i> , <i>CD8</i> , CD3G , <i>CDK16</i> , <i>CSNK1A1</i> , <i>DAPK1</i> , <i>EFNA1</i> , <i>EFNA3</i> , <i>EFNA4</i> , EPHA6 , <i>EPHA</i> , <i>FLNC</i> , <i>FSH</i> , <i>GZMA</i> , <i>HMGB2</i> , <i>IL3</i> , <i>IL4</i> , <i>Lh</i> , <i>Mapk</i> , <i>MKNK2</i> , <i>OSM</i> , <i>OTUD1</i> , PDZK1IP1 , <i>PPP1R14A</i> , <i>PSMG1</i> , <i>RAD23A</i> , <i>STK25</i> , <i>TMEM97</i> , <i>TP53</i> , <i>ZBTB2</i>	19	11	Cell-To-Cell Signaling and Interaction, Lipid Metabolism, Small Molecule Biochemistry

The genes found to be differentially regulated in our experiments and the number of such genes displayed in the “Focus Molecules” column have been highlighted in **bold print** that meet the criteria cutoff and/or filter criteria, and were mapped to its corresponding gene object in IPA Knowledge base (*Italicized Bold*= Up-regulated; *Italicized* = Down-regulated). The score is generated using a p-value calculation. This score indicates the likelihood that the assembly of a set of focus genes in a network could be explained by random chance alone. The data base attributed general cellular functions to each network which are determined by interrogating the Ingenuity Pathway Knowledge base for relationships between the genes in the network and the cellular functions they impact.

Table 4

Differential expression of 14 genes of interest through relative quantification (Ct) that selected for high-throughput TLDA card design and their corresponding Probe sets (pre- designed and validated, from ABI, CA) in *in vitro* and small set population validation study.

Gene Name (Probe Sets)	Descriptions/Functions	In Vitro Results (n=6)*	Population Results (n=71)*
APC (<i>Hs01568270_m1</i>)	An antagonist of the Wnt signaling pathway	0.33 (–)	0.62 (–, n=53, 96%)
ARNT (<i>Hs01121918_m1</i>)	Fusin protein associated with acute myeloblastic leukemia	0.32 (–)	0.56 (–, n=52, 94%)
CD3G (<i>Hs00173941_m1</i>)	Gamma polypeptide (TIT3 complex), Immunodeficiency	0.31 (–)	0.64 (–, n=53, 96%)
CYP1A2 (<i>Hs01070374_m1</i>)	A member of Cytochrome P450 superfamily enzyme	0.47 (+)	NV
CYP2D6 (<i>Hs02576168_m1</i>)	A member of Cytochrome P450 superfamily enzyme	0.47 (–)	0.45 (–, n=32, 57%) 0.22 (+, n=19, 33%)
ENTPD3 (<i>Hs00928977_m1</i>)	Ectonucleoside triphosphate diphosphohydrolase 3, Catabolism of extra cellular nucleotide	0.72 (–)	1.09 (–, n=50, 90%) 0.30 (+, n=4, 7.0%)
ITGB1 (<i>Hs01127543_m1</i>)	Integrin, beta 1 (fibronectin receptor, beta polypeptide)	0.27 (–)	NV
LEPR (<i>Hs00174492_m1</i>)	Leptin receptor (Obesity)	0.36 (–)	0.66 (–, n=52, 94%) 0.12 (+, n=3, 5.0%)
LPR12 (<i>Hs00257526_s1</i>)	Low density lipoprotein –related protein 12; candidate tumor suppressor gene	0.32 (–)	0.67 (–, n=52, 94%) 0.30 (+, n=3, 5.4%)
MYC (<i>Hs00153408_m1</i>)	Proto-oncogene, cell cycle progression, apoptosis and Transformation/transcription of specific target gene	0.39 (–)	0.73 (–, n=52, 98%) 0.10 (+, n=1, 1.8%)
RRAD (<i>Hs00188163_m1</i>)	Ras-related associated with diabetes	0.29 (–, n=4) 0.25 (+, n=2)	0.23 (–, n=10, 14%) 0.54 (+, n=60, 86%)
TAB1 (<i>Hs00196143_m1</i>)	TGF-beta activated kinase 1/ MAP3K7 binding protein 1	0.27 (–)	0.82 (–, n=54, 76%) 0.80 (+, n=16, 24%)
TP53 (<i>Hs01034249_m1</i>)	Tumor protein p53	0.34 (–)	0.40 (–, n=51, 71%) 0.28 (+, n=15, 21%)
TRAP1 (<i>Hs00212476_m1</i>)	TNF receptor-associated protein 1	0.31 (–)	0.64 (–, n=52, 74%) 0.14 (+, n=3, 4%)

18s –RNA (*Hs99999901_s1*) Manufacturing Control;

GAPDH (*Hs99999905_m1*) Glyceraldehyde-3-phosphate dehydrogenase (Inter. Control)

NV - Not Validated

* Total Number of Subjects in this population based pilot observation

** Data represented as Ct changes (relative quantification) with Down-regulation (–)/Up-regulation (+). Number (n) in parenthesis is the total number of subjects where such changes were observed. % calculation was made only among the subjects with amplification under this validation platform.