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Meta-analysis of Microarray Datasets for the Risk Assessment of Coplanar Polychlorinated Biphenyl 77 (PCB77) on Human Health

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Received 2 June 2017 / Received in revised form 13 June 2017
Accepted 16 June 2017
DOI 10.1007/s13530-017-0317-1

pISSN : 2005-9752 / eISSN : 2233-7784
Toxicol. Environ. Health. Sci. Vol. 9(2), 161-168, 2017

Electronic supplementary material
The online version of this article (doi:10.1007/s13530-017-0317-1) contains supplementary material, which is available to authorized users.

Abstract

Polychlorinated biphenyls (PCBs) are persistent organic compounds that have been banned since 1970s, but continue to contaminate the environment. PCBs are categorized into two structural groups: coplanar and non-coplanar PCBs. The coplanar PCBs are dioxin-like potent toxic compounds. To evaluate their effects on humans, we chose a coplanar PCB77 for data analysis. We performed meta-analysis by integrating datasets via the Rank Product method, and identified 375 up- and 66 down-regulated differentially expressed genes (DEGs). Notably, up-regulated genes were significantly associated with liver and kidney diseases. Using gene ontology enrichment, we found that the up-regulated DEGs were significantly enriched in the apoptotic process (false discovery rate, FDR = 1.62e-10) and response to unfolded protein (FDR = 7.65e-10). Protein-protein interaction networks identified the hub proteins containing HSP90AB1 and HSPA5. These findings suggest that our DEGs may provide a robust set of genetic markers for PCB77.

Keywords: Coplanar Polychlorinated biphenyls, Meta-analysis, Risk assessment, Liver disease, Kidney disease

Introduction

Polychlorinated biphenyls (PCBs) are organic chlorine compounds with 1 to 10 chlorine atoms attached to a biphenyl ring [$C_{12}H_{10-n}Cl_n$ (n = 1-10)] (Figure 1). Due to their inherent properties of high thermal conductivity, low flammability, and high resistance to thermal degradation[1], PCBs were widely used as dielectric fluids in capacitors and transformers, heat transfer fluids, hydraulic fluids, coolants, lubricants, and sealants, as additives in paints, plastics, and dyes, and as extenders in pesticide. PCBs are endocrine disruptors which act as xenoestrogens that accumulate in the body fat, and are usually amassed through contaminated food and water. Most recently, it was found that one of UK's last killer whales (LuLu) had shockingly high levels of PCB contamintion[2]. Previous studies showed that PCBs act as carcinogens in breast[3], stomach[4], and liver[5,6], and are also implicated in atherosclerosis and cardiovascular diseases[7]. When fetus or newborns are exposed to PCBs, they may retain permanent and irreversible damages[8,9] in developing nerves and reproductive organs[10].

Theoretically, a total of 209 isoforms or congeners of PCBs can be produced, which are further subdivided into 10 homologs, depending on their characteristics[11]. There are a total of 12 types of coplanar PCBs and rest of them are all in the categorized as non-coplanar PCBs. Non-coplanar PCBs have chlorines in the *ortho*-position, whereas coplanar PCBs have chlorine atoms in both *para*-positions and at least one in the *meta*-position, but are lacking in the *ortho*-position (Figure 1)[12,13]. Coplanar PCB structures are analogous to dioxins because of rotation of phenyl-phenyl groups. It has been shown that traces of coplanar PCBs impart the toxicity to mixed PCBs[1].

The microarray technology has been used to identify the impact of various chemicals on animals, especially humans. The public microarray databases such as NCBI GEO, ArraryTrack, and ArrayExpress have been consistently accumulating quantified and standardized experimental data. In addition, bioinformatics tools have become more diverse, statistical techniques more powerful, thus enabling the integration of heterogeneous platforms from different studies and selecting

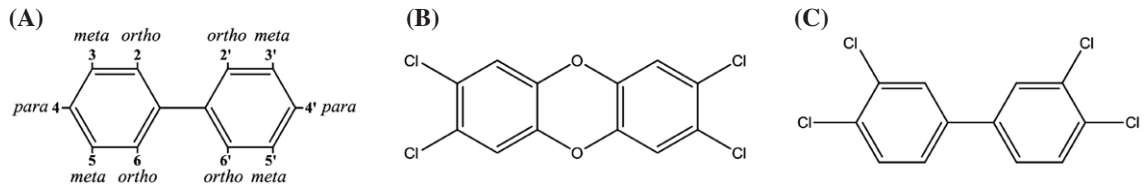

Figure 1. The nomenclatures and structures of PCBs, dioxin, and PCB77. (A) The structure of PCBs. There are biphenyl groups with 12 carbons, forming four *ortho*-, four *meta*-, and four *para*-positions. (B) The structure of tetrachlorodibenzodioxin, one of the representative dioxins. (C) The structure of a coplanar PCB, PCB77.

differentially expressed genes (DEGs) with increased statistical power[14]. Because it has been widely reported that the toxic response of coplanar PCBs may vary greatly depending on the species and organs, it is difficult to undertake cross-species analysis between different animal species. In addition, non-coplanar PCBs are often contaminated by coplanar PCBs, and the members of coplanar PCBs may have different modes of toxic reactions. Thus, we chose to work with microarray data derived from human samples treated with a single compound of coplanar PCB, namely PCB77[15]. In this study, we used the Rank Product algorithm[16,17] to identify DEGs, using microarray data obtained from PCB exposure experiments in human cells. The gene expression levels of meta-analysis were compared with four liver and two kidney disease gene sets using Gene Set Enrichment Analysis (GSEA)[18]. Gene ontology (GO) enrichment analysis and Protein-protein interaction (PPI) networks were conducted to identify whether the levels of gene expressions with PCB77 exposure were associated with liver and kidney diseases.

Results and Discussion

PCBs have once been widely used for many products, including transformers and hydraulic fluids, and coolants[11]. After their environmental toxicity were confirmed, the production was banned in many countries. However, due to their chemical stability, PCBs are still detected in the environment in the current days and they are considered as persistent organic pollutants (POP). All PCBs induce the formation of reactive oxygen species, genotoxic effects, immune suppression, inflammatory response, and endocrine effects[19] to various extents and through different pathways[20]. Of the 206 possible congeners of PCBs, 12 PCBs are categorized as coplanar PCBs[11]. Dioxin and dioxin-like chemicals are a family of diverse toxic chemicals with a similar chemical structure, and sharing a common mechanism of toxicity[21]. Coplanar PCBs are considered to be dioxin-like and are of special interest since they can affect humans through Aryl hydrocarbon receptor (AhR) activation, similar to the dioxin family. These effects are mainly related to carcinogenesis due to their involvement in cell cycle control[22], cell proliferation[22], inhibition of apoptosis[23], and suppression of cell-to-cell communication[24]. In this study, we attempted to investigate the effects of coplanar PCBs on humans via meta-analysis for the first time. After integrating datasets related to coplanar PCB77 exposure to human cells and adjusting for batch effects, Rank product algorithm was used to identify DEGs.

Meta-analysis to Identify Differentially Expressed Genes

Recent studies showed that dioxin-like compounds such as coplanar PCBs vary in their modes of toxic response amongst the different species. Therefore, we originally obtained all the available datasets from the NCBI GEO database, where human cells were exposed to single compound coplanar PCBs. These datasets were obtained from the human cell lines, such as PCB77 on human liver carcinoma HepG2 cells (GSE6869), PCB77 on human kidney cells HK2 cells (GSE23493), and PCB126 on human primary hepatocytes (GSE 14553). However, as claimed by the original study and confirmed by our analysis, the responsivity of human hepatocytes to PCB126 was very low (data not shown)[25]; hence, the dataset GSE14553 was eliminated from our study. In addition, since the studies revealed that the expression levels of many genes are not significantly regulated at 30 min of PCB77 exposure[26], these data were eliminated from the final analysis. A limitation of this study is that the datasets used in this study is very small compared with other meta-analysis research. This phenomenon may possibly be due to the early (ca. 1970) ban of PCBs in many countries. In addition, the modes of toxic responses are known to be very different in other animals so that it was impossible to make cross-species analyses[25].

To identify robust genetic markers for the risk assessment of PCB77, we first converted microarray probe IDs to unique Entrez gene IDs using the average value of several probes. A total of 20,514 genes were obtained in the two datasets we used. After adjusting for batch

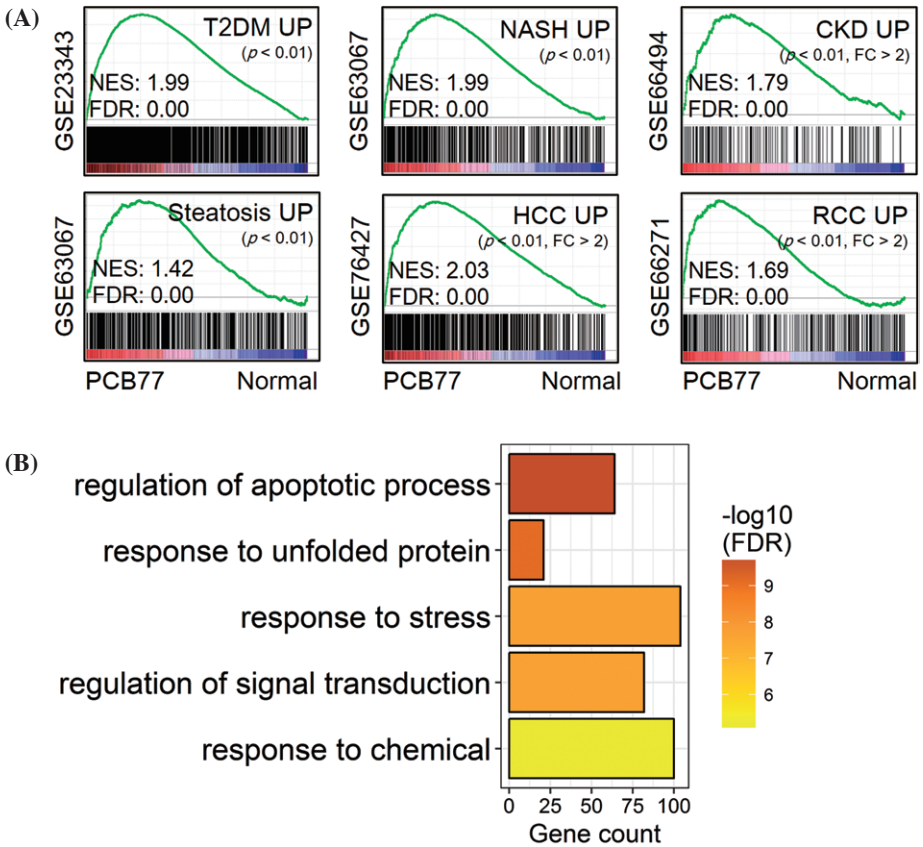

Figure 2. Functional enrichment analysis and the correlation between PCB77 and liver and kidney diseases. (A) GSEA plots showing that up-regulated DEGs of four liver and two kidney diseases were significantly enriched with PCB77 exposure. The GEO accession number was shown on the left side of each GSEA plot. (B) Functional enrichment analysis of GO biological process terms using up-regulated DEGs under PCB77 exposure. The color represents significance levels. T2DM, type 2 diabetes mellitus; NASH, non-alcoholic steatohepatitis; HCC, hepatocellular carcinoma; CKD, chronic kidney disease; RCC, renal cell carcinoma; NES, normalized enrichment score; FDR, false discovery rate.

effects, we performed meta-analysis on the integrated datasets to identify DEGs using the Rank Product (RP) method, which is a robust algorithm for meta-analysis using multiple heterogeneous datasets. By applying a minimal fold change (FC) of 1.2 and a cutoff *p*-value of 0.01, a total of 441 genes were identified as DEGs. 375 genes were up-regulated and 66 genes were down-regulated under PCB77 treatment (Table S1).

Identifying the Correlation between Diseases and PCB77

In humans, it is well established that the most profoundly affected organ of short-term exposure to dioxins and dioxin-like compounds is the liver[27]. In addition, kidney is one of the extrahepatic tissues which is in direct contact with such xenobiotic agents during metabolism and clearance[28]. In order to understand whether the exposure to PCB77 was related to disease states at the gene expression levels, GSEA was conducted using microarray datasets from liver and kidney diseases in humans. The gene sets DEGs of type 2 diabetes (T2DM) of GSE23343[29], steatosis, non-alcoholic steatohepatitis (NASH) of GSE63067[30], hepatocellular carcinoma (HCC) of GSE76427, kidney tissue on renal cell carcinoma (RCC) of GSE66271[31], and chronic kidney disease (CKD) of GSE66494[32] were identified using the limma R package.

The GSEA results showed that the up-regulated DEGs obtained from four liver and two kidney diseases were significantly enriched in up-regulated when PCB77 (Figure 2A). Because the up-regulated DEGs were closely associated with various liver and kidney diseases, the DEGs were assigned to the GO enrichment analysis to gain insights into the biological roles of PCB77 exposure. We found that our up-regulated DEGs were significantly enriched in the regulation of apoptotic process (GO: 0042981, FDR = 1.62e-10), response to unfolded protein (GO: 0006986, FDR = 7.65e-10),

response to stress (GO: 0006950, FDR = 1.90e-08), regulation of signal transduction (GO: 0009966, FDR = 2.08e-08), and response to chemicals (GO: 0042221, FDR = 6.85e-06) (Figure 2B).

Even though both non-coplanar and coplanar PCBs induce oxidative stress and eventually apoptosis, they use distinctly different pathways[26]. While the oxidative stress induced by non-coplanar PCBs activates the Fas receptor signaling pathway, coplanar PCBs, along with dioxin and dioxin-like chemicals, have a strong association with aryl hydrocarbon receptors (AhR). The main role of AHR pathway is to increase the expression of cytochromes P450 1A1 (CYP1A1), which in turn hydroxylates the chemical when a xenobiotic enters the body. CYP1A1 detoxifies polycyclic aromatic compounds and the activation of this enzyme generates mutagenic metabolites and oxidative stress[33]. Binding affinities differ for the AhR, depending on the chemical structures of compounds. The binding affinity of dioxin is the highest, followed by coplanar PCB, and non-coplanar PCB having the lowest binding affinity. The expression levels of AhR-dependent CYP1A1 increased in a dose-dependent manner when treated with dioxin[34]. These results were consistent with our finding that CYP1A1 (FC = 4.25, p = 1.7.E-09) was the most up-regulated DEG. Notably, AhR (FC = 1.42, p = 1.67e-02) was not classified as a DEGs in our study, since we used a very strict threshold ($p <$ 0.01). However, when the significance was set at 0.05, the expression level of AhR can be considered as significant. Additionally, MYC (MYC proto-oncogene, bHLH transcription factor), and NDRG1 (N-myc downstream regulated 1), which are closely related to the AHR pathway[26], were also up-regulated DEGs under PCB77 exposure (Table S1).

Over 70% of patients with T2DM have had NAFLDs, which in extreme cases can develop into serious liver disorders, such as NASH, liver cirrhosis, and HCC[35]. Our results showed that PCB-treatment is significantly correlated with these liver disorders in up-regulated genes in response to PCB exposure (Figure 2A). In addition, similar correlation patterns were observed in the expression datasets of kidney diseases, including CKD and RCC (Figure 2A). To determine the biological processes involved, the GO enrichment analysis was performed using up-regulated DEGs. Our top 3 significantly enriched GO terms were the regulation of apoptotic process (FDR = 1.62e-10), response to unfolded protein (FDR = 7.65e-10), and response to stress (1.90e-08) (Figure 2B). All three terms were in close association with endoplasmic reticulum (ER) stress[36]. Even though the long-term exposure of dioxin and dioxin-like xenobiotics are known to interfere with the development of brain and reproductive organs in fetus[37], we were unable to detect any meaningful correlation with the down-regulated genes in GSEA as well as GO enrichment analysis in our study. This may be due to the fact that our datasets were mainly obtained from adult human cells with a short-term exposure to PCB77 and the number of samples. Together, the data suggests that the main target disease development and/or pathogenesis of PCB77 are via up-regulated genes.

Protein-protein Interaction Network Analysis

Because the expression levels of the up-regulated DEGs were significantly related to liver and kidney diseases, the protein-protein interaction (PPI) networks were established for understanding the biological interactions among the up-regulated genes. In the entire PPI network (Figure 3), a sub-network was identified using DEGs contained in 5 GO terms, as shown in Figure 2B. The network consisted of 50 proteins and 60 interactions (Figure 4). Among the total 50 proteins, 23 proteins were involved in regulation of apoptotic process GO term (Figure 2B). Notably, the hub proteins containing HSP90AB1 (heat shock protein 90 alpha family class B member 1, Degree = 10) and HSPA5 (heat shock protein family A (Hsp70) member 5, Degree = 9) were also included in response to unfolded protein GO term (Figure 2B). The HSP90 dimer binds with Ahr protein before the AHR signaling pathway[38]. A transgenic mouse with constitutively active Ahr caused by removing the HSP90-binding domain (amino acid residues 288-421) promoted hepatocarcinogenesis[39]. HSPA5, an ER chaperone protein, is a central regulator of ER stress, involved in folding of the new proteins and refolding of damaged proteins, and thereby regulating the stability between cell survival and apoptosis in ER-stressed cells[40,41]. Collectively, PPI network analyses showed that HSP90AB1 and HSPA5 were major proteins implicated in liver and kidney toxicity of PCB77.

This study is not without limitations[20]. We were unable to carry out experiments for validation of PCB77-induced genes since import of PCB has been banned in Korea since 1996. The amount of the datasets used in this study was very small, possibly due to the early ban of PCBs. This situation is not likely to improve in future, due to the almost global ban of PCB production. Various studies are still performed with PCB-contaminated animals and human subjects. However, the results cannot provide definitive answers for the risk assessment of specific PCBs, because the subjects are exposed to various mixtures of PCBs. At this point, we believe that our study is the best possible meta-analysis of the risk assessment of PCB77 in human health.

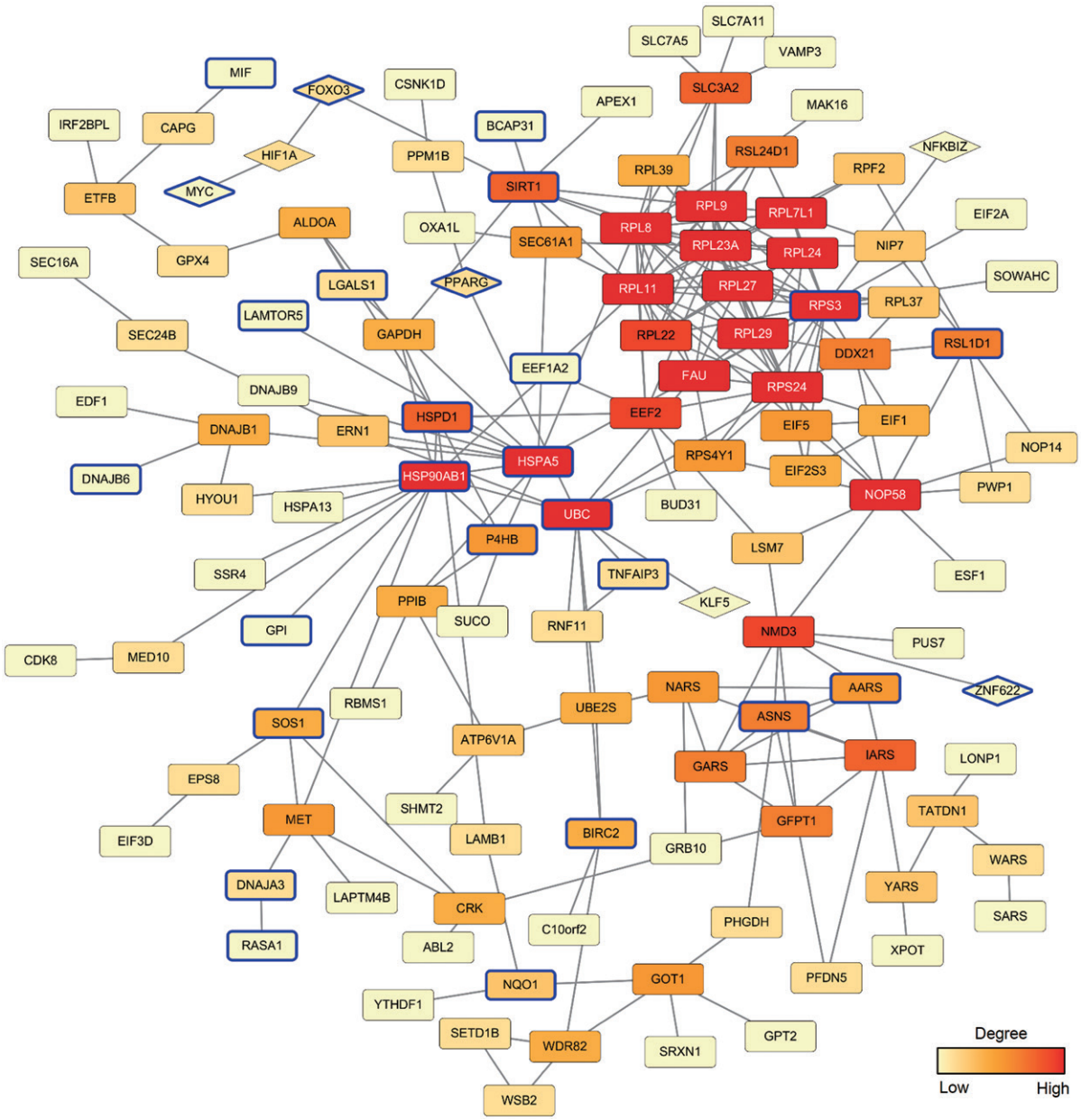

Figure 3. The entire protein-protein interaction network of the up-regulated DEGs. 124 nodes represent proteins and 249 edges represent interactions. Proteins are indicated as round rectangles, and transcription factors as diamonds. The node color represents the neighborhood connectivity (degree). The blue border represents the proteins involved in the regulation of apoptotic process (GO: 0042981) GO term in Figure 2B.

Materials and Methods

Data Mining to Identify Genes Associated with PCB Exposure

The NCBI Gene Expression Omnibus (GEO) database[42] was used to obtain raw data for meta-analysis. The obtained data were from the Affymetrix microarray datasets associated with coplanar PCBs, with a clear note that coplanar PCBs were treated as a single compound. Coplanar PCBs are known to be similar to dioxins in terms of chemical structure and mode of action in animals. The obtained datasets were two GEO series (GSE): PCB77 on human liver carcinoma HepG2 cells (GSE6869) and on human kidney cells HK2 cells (GSE23493). The total number of control groups was 6 and that of PCB77-treatment groups was

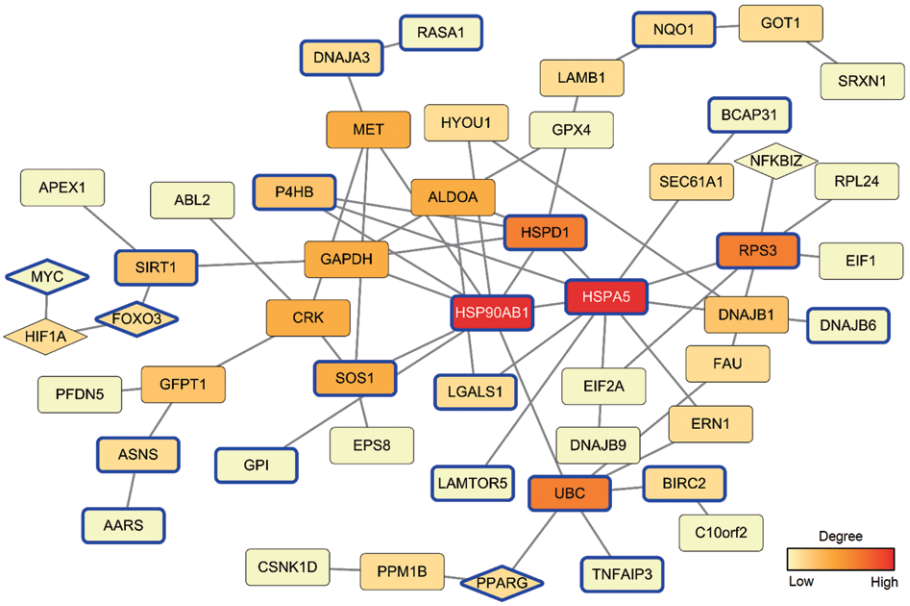

Figure 4. A protein-protein interaction network of the up-regulated DEGs in five major GO terms. Nodes represent proteins and edges represent interactions between two proteins. Proteins are indicated as round rectangles, and transcription factors as diamonds. The node color represents the neighborhood connectivity (degree). The blue border represents the proteins involved in regulation of apoptotic process (GO: 0042981) GO term in Figure 2B.

12, in which 30 min-treated samples were excluded.

Data Pre-processing

The raw datasets of each microarray GSE were processed by the *oligo* package[43] of R language, and the expression values were normalized by the RMA (robust multi-array average) algorithm[44,45]. All microarray probe names were replaced with unique Entrez ID using the Human Affymetrix microarray annotation package, *hgu133plus2.db*[45], because Entrez IDs are the universally used IDs in numerous bioinformatics analysi. In case where multiple values were obtained for a single Entrez ID, the arithmetic mean of the values was used as the gene expression value.

Adjusting for Batch Effects

Adjusting for non-biological batch effect was performed following Jung *et al.*[45]. Briefly, the surrogate variable analysis (*SVA*) packages in R language was used to remove batch effects which may occur from non-biological variation arising from merging multiple studies performed in different environments[45]. Within this package, the ComBat function, which adjusts each gene expression level independently[46], was used to remove batch effects.

Differentially Expressed Gene Analysis Using Rank Product Methods

The R package RankProd was used to find DEGs in the integrated datasets as previously described[45]. Based on the ranked fold change (FC) of each gene, the Rank Product (RP) values were calculated by RP function in the RankProd R package. With this RP value, DEGs were allocated to the two lists of up-regulated and down-regulated genes through default 100 permutation tests.

Gene Set Enrichment Analysis (GSEA)

The GSEAPreranked method was used for the GSEA. Gene expression datasets for human liver tissues on type 2 diabetes (GSE23343)[29], steatosis, non-alcoholic steatohepatitis (GSE63067)[30] and hepatocellular carcinoma (GSE76427) were used, as well as for human kidney tissues on renal cell carcinoma (GSE66271)[31] and chronic kidney disease (GSE66494)[32]. DEGs derived from the limma R package[47] were used as gene sets.

Gene Ontology Enrichment and Protein-protein Interaction Network Analysis

The protein-protein interactions (PPI) network and gene ontology (GO) enrichment analysis was performed using the STRING database (v10.5)[48]. Interaction sources in this database are divided into 5 categories: (1) experimental interactions, (2) pathway knowledge interactions obtained from manually curated databases, (3) text-mining interactions using a large collection of articles, (4) predicted interactions using genomic information and co-expression analyses, and (5) inter-

actions of orthology relations that are systematically applied to other organisms, when the observation was made in one organism. In this study, only experimental interactions were used. The PPI network was visualized by Cytoscape software[49]. The GO enrichment analysis was conducted on biological processes.

Acknowledgements

This work was supported by the National Research Foundation of Korea (NRF) grant funded by the Korea Ministry of Science, ICT & Future Planning (MSIP) (Grant No. 2015R1C1A2A01054400).

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