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Reduced expression of von Hippel–Lindau gene in subjects exposed to polychlorinated biphenyls and dibenzofurans ☆

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ABSTRACT

Polychlorinated biphenyls (PCBs) and polychlorinated dibenzofurans (PCDFs) are ubiquitous pollutants found in the environment and human tissues. A cohort in Taiwan has undergone follow-up for 24 years after exposure to high levels of PCBs and PCDFs. The incidence of chloracne, hyperkeratosis, and abnormal nail was increased among exposed people. We conducted a study to identify the genes whose expressions were affected by such exposure. A cDNA microarray system consisting of 908 genes was used for pooled serum samples from non-smoking men exposed to PCBs and PCDFs ($n = 15$) and their matched referents ($n = 15$) in triplicate. After adjusting for background and housekeeping genes, genes with different expressions between the exposure and reference groups were determined by both regression and cluster analysis, and further confirmed by real-time RT-PCR. The tumor suppressor gene von Hippel–Lindau (VHL) was found to be down-regulated in the microarray analysis. VHL gene expression levels were also found to be positively associated with age, shown by real-time RT-PCR. Upon age adjustment, VHL gene expression was reduced in Yucheng ("oil disease") subjects as compared to referents. Among Yucheng people, those with abnormal nails had lower VHL expressions than those without abnormal nails. These findings provide new insights into the potential role of VHL in health conditions associated with PCB and PCDF exposures.

1. Introduction

Polychlorinated biphenyls (PCBs) are ubiquitous environmental pollutants, which became recognized as potentially harmful environmental contaminants in the 1960s. Polychlorinated dibenzofurans (PCDFs) and polychlorinated dibenzodioxins (PCDDs) are byproducts of human activities, including manufacturing, farming, and incineration. Despite the ban on PCBs in many countries in the 1970s and cautious monitoring and control of dioxin-like chemicals, these chemicals likely will stay in the environment and food chain for many years to come due to their chemical stabilities (Kimbrough, 1985).

In 1979, a mass poisoning of more than 2000 people occurred in central Taiwan due to the consumption of rice-bran cooking oil contaminated with PCBs and PCDFs (Hsu et al., 1985). The exposed people, later called Yucheng ("oil disease" in Chinese) victims, suffered from chloracne, hyperpigmentation, peripheral neuropathies, liver function abnormalities, and other signs and symptoms (Guo et al., 1999). The victims were estimated to have consumed an average of 1 g of PCBs and 3.8 mg of PCDFs in total over an average of 9 months of exposure to the contaminated oil (Lan et al., 1981). Such consumption resulted in a median serum level of 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin toxic equivalency (TEQ) approximately 100 times higher than the background-exposed reference group immediately following exposure (Guo et al., 1997; Lambert et al., 2006). Sixteen years after exposure, TEQ in the Yucheng population remained 20–40 times higher than in the reference population (Lambert et al., 2006).

Recent advances in molecular technology have allowed the study of more subtle effects of environmental exposure to PCBs and PCDFs, as well as their biomarkers. Among them, cDNA microarray technology was one of the most powerful tools for screening thousands of genes for expression levels simultaneously (Butte, 2002). The following real-time RT-PCR approach could

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verify the affected gene expressions identified in the microarray analysis (Chuaqui et al., 2002; Petricoin et al., 2002). With information about the health outcomes associated with PCB and PCDF exposure (Guo et al., 1999), the relationship between health outcomes and gene expression can also be examined.

2. Materials and methods

2.1. Subjects and samples

A total of 15 Yucheng and 14 male referents matched on age, gender, and neighborhood (same village) donated 30 ml of venous blood (Table 1). To avoid the potential effects of cigarette exposure and hormone condition on gene expression profiles, we recruited only non-smoking men in this study. Initially, a total of 29 subjects donated blood for the screening microarray study. Peripheral lymphocytes were isolated from the whole blood immediately, and total RNA was extracted. These samples were pooled to avoid any specific sample biases for microarray analysis. In a follow-up health examination, additional 14 subjects donated blood and a total of 43 subjects were included in real-time RT-PCR analysis. These analyses were done on specimens from individual subjects and not pooled materials.

2.2. cDNA microarrays to screen gene expressions

2.2.1. Microarray chip

The Millennium® cDNA nylon membrane microarray chips used in this study were designed and manufactured by The Center for Microarray at the National Cheng-Kung University School of Medicine, Tainan, Taiwan. On each chip, 908 human cDNAs were identified, including stress response factors, DNA repair genes, tumor suppressors/oncogenes, cell cycle regulators, kinases, proteases, and transcriptional factors. Fourteen endogenous housekeeping genes were used for data normalization (Guo et al., 2002).

2.2.2. Labeling of cDNA and hybridization to arrays

Cellular mRNAs underwent reverse transcription to cDNAs, and were labeled with biotin for hybridization reactions. An equal number of mRNA samples were pooled by exposure status. The protocol for labeling was previously described in our study (Guo et al., 2002). Visualization of the hybridized signals was also performed in accordance with our previous study (Guo et al., 2002). Triplicate analysis of each assay condition was performed.

2.2.3. Data collection and normalization

The color images were analyzed using a high-resolution scanner (PowerLook 3000; UMAX Data Systems, Inc., Taipei, Taiwan). The hybridization signals were quantified with ScanAlyze v2.5 software (Eisen Lab, Stanford University, Stanford, CA). The software digitizes the signal intensity of each spot and the background level around it. The intensity of each spot was divided by its background level to adjust the level of brightness of each probe. The ratio of signal intensity was adjusted by the geometric mean of the 14 housekeeping genes of the six treatments (two groups in triplicates). The ratio expression was then analyzed as raw data in further statistical analyses.

2.2.4. Statistical analysis for microarray data

In this study, data were analyzed by JMP 5.0 of the SAS statistical packages (SAS Institute Inc., Cary, NC). Cluster and regression analyses were used to detect gene expressions. First, we used the raw data minus the minimum expression ratio round off to the 2nd decimal place (0.76) to shift the baseline to close to zero and a natural log transformation to normalize the distribution. Under general assumption, the slope of the line, approximately 1, meant there was no significant difference in gene expression between the exposure and reference groups. A regression line and 95% ellipse prediction interval were calculated to detect genes outside the interval (Fig. 1). Second, hierarchical clustering was used to identify samples with a similar pattern of genes. The two closest expressions were clustered together until all expressions were grouped into a single cluster. The clustering algorithm calculated the distance between the two genes by "Cluster" program and plotted the correlation of genes by "Cluster Treeview" program (Fig. 2) (Eisen et al., 1998).

2.3. Quantitative real-time RT-PCR to validate the microarray data

2.3.1. Quantitative real-time RT-PCR protocols

We used LightCycler® (Roche Applied Science, Roche Diagnostics Corp., Indianapolis, IN) in combination with the dsDNA-binding dye SYBR Green I for quantification. The expression of the von Hippel–Lindau (VHL) gene was quantified by measuring the fractional cycle number at which the level of expression reached a fixed threshold and was directly related to the amount of product. PCR products of known concentration were used to construct a standard curve. The housekeeping gene GAPDH, which maps to 12p13, served as an internal control to normalize the results for variations of input RNA amount or efficiency of reverse transcription (Thellin et al., 1999). The primer used for GAPDH gene was 5′-TGAAGGTCGGAGT CAACGGATT-3′ (forward) and 5′-CCTGGAAGATGGT GATGG-GATT-3′ (reverse), and for VHL was 5′-CTGCCCG TATGGCTCAA CTT-3′ (forward) and 5′-GTGTGTCCCTGC ATCTCTGAAG-3′ (reverse).

Total RNA was isolated from lymphocyte samples and reversely transcribed using oligo $(dT)_{15}$ primer and MMLV reverse transcriptase. The cDNA products were subjected to PCR reactions following the LightCycler® format. The 10-μL PCR reaction mix contained the following reagents: 3 mM of $MgCl_2$, 0.2 μM of each primer, 1 μL of LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics, Indianapolis, IN), and 1 μL of cDNA. The PCR reaction used the following sequence: 95 °C for 10 min, then 45 cycles with each cycle consisting of 95 °C for 5 s, 60 °C for 5 s, and 72 °C for 10 s. Finally, the PCR tubes were incubated at 72 °C for 10 min. The

Table 1
Age distribution, alcohol use, dermatologic manifestations, and PCB concentrations in study and control groups in microarray analysis and real-time RT-PCR

Microarray analysis	Exposure ($n = 15$) [N (%)]	Reference ($n = 14$) [N (%)]	P-value[a]
Age (year)	44.2 ± 1.6[b]	49.8 ± 1.7	0.02
30–39	5 (33.3)	0 (0.0)	0.001
40–49	9 (60.0)	6 (42.9)	
50–59	1 (6.7)	8 (57.1)	
Ever alcohol use	6 (40.0)	6 (42.9)	0.88
PCB concentration (ppb)	58.5 (7.0–134.0)	1.7[c]	–
Real-time RT-PCR	Exposure ($n = 28$)	Reference ($n = 15$)	P value[a]
Age (year)[b]	43.1 ± 7.3	48.3 ± 9.5	0.05
PCB concentration (ppb)	63.0 (7.0–459.0)[d]	1.7[c]	–
Skin problems	Exposure ($n = 11$) [N (%)]	Reference ($n = 7$) [N (%)]	P value[a]
Age (year)	46.1 ± 5.6	51.0 ± 4.9	0.08
Chloracne	5 (54.6)	0 (0.0)	0.02
Abnormal nail	6 (54.6)	0 (0.0)	0.01
Hyperkeratosis	3 (27.3)	0 (0.0)	0.1
PCB concentration (ppb)	103.0 (7.0–317.0)[d]	1.7[c]	–

[a] Twenty-eight exposed and 15 reference subjects were analyzed by real-time RT-PCR. Among them, only 11 exposed and eight reference subjects completed the questionnaire on medical conditions in 2003.
[b] Mean ± standard deviation.
[c] General population had mean serum PCB levels of 1.67 ppb wet weight (Guo et al., 1997).
[d] Mean PCB levels (minimum–maximum).

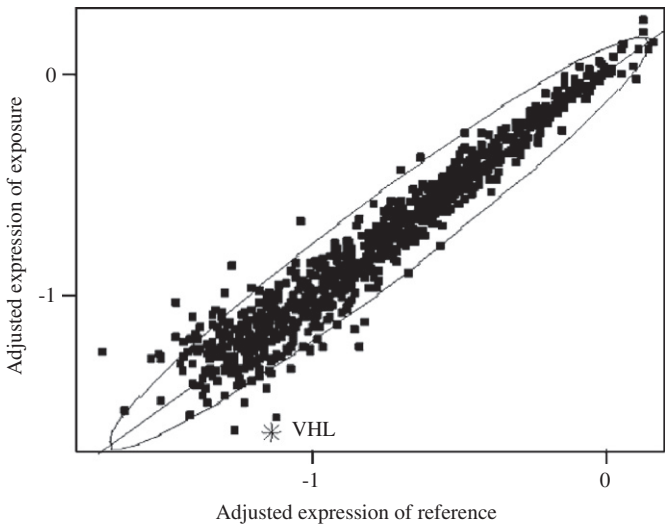

Fig. 1. Regression analysis of the 908 target gene expressions in the subjects of exposure and reference groups. The regression equation was log (expression of exposure −0.76)−0.00110.984 log (expression of reference −0.76), $R^2 = 0.94$, $P < 0.0001$. Gene expression in plot was adjusted for baseline and log transformation. The ellipse range was the 95% prediction interval based on the regression line.*: VHL gene was consistently found down-regulated in exposure group by regression and cluster analyses.

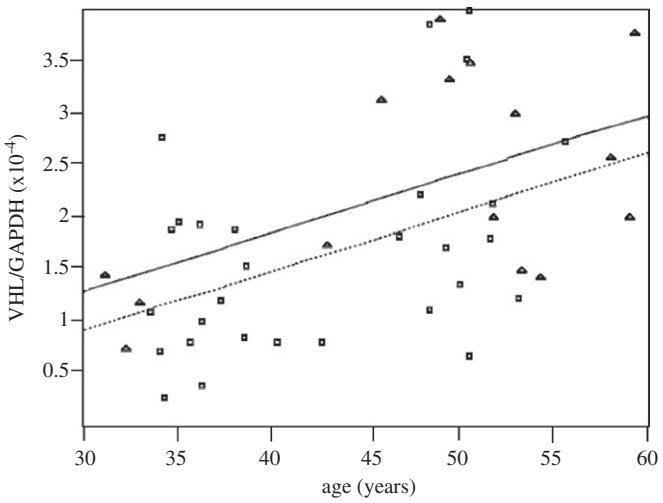

Fig. 3. Association between the levels of PCB exposure and VHL expression adjusted by age. The VHL expression was significantly different between exposed and reference subjects (0.00017 ± 0.00002 vs. 0.00023 ± 0.00003, $P = 0.04$). The top line across the graph represents VHL expressions in control subjects (triangles) ($n = 15$), and shows a positive association between VHL expression and age ($P = 0.046$). The lower line represents VHL expression in exposed subjects (squares) ($n = 28$), and also shows a positive association between VHL expression and age ($P = 0.023$).

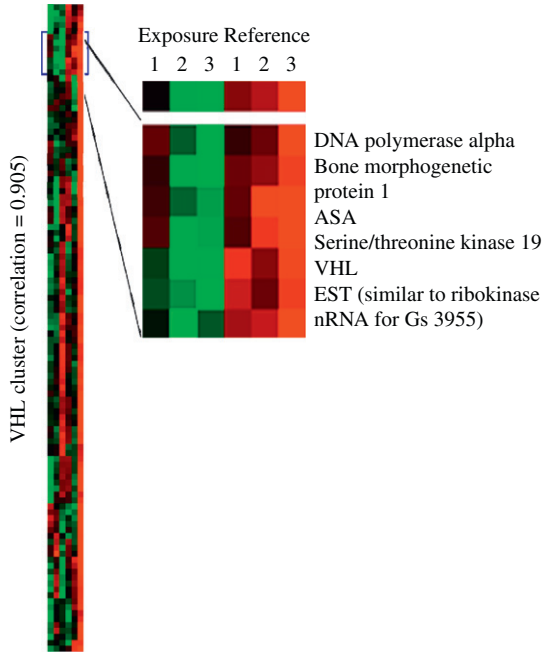

Fig. 2. Profile of gene expressions by cluster analysis in exposure and reference groups. Green and red colors mean gene down- and up-regulations, respectively. Darkness of each color is associated with the level of gene expression. The figure is partial representation of 908 gene expressions due to space limitation.

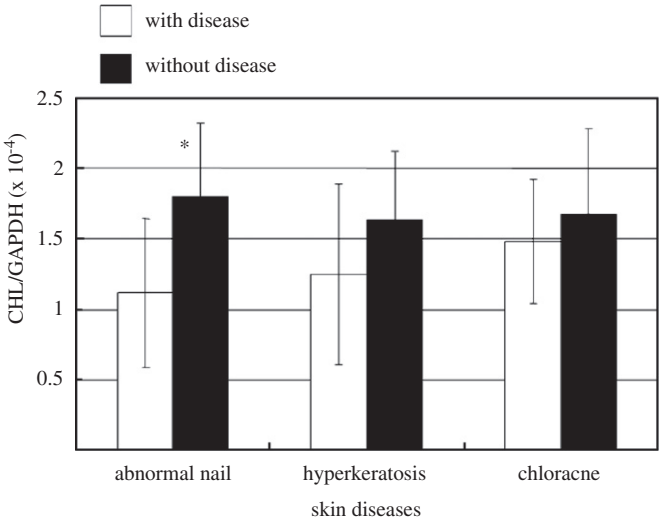

Fig. 4. The association between VHL expression and skin problems in Yucheng subjects ($n = 11$). The expression level of VHL was normalized with that of GAPDH in each subject. Open bars represent the Yucheng subjects with skin problems; closed bars represent those without skin problems. *$P < 0.05$ (with disease vs. without disease).

intensity of fluorescence emitted by SYBR Green I was detected at the end of each PCR cycle.

To determine copy numbers of the target transcripts, an external standard curve was calculated for VHL. The copy numbers of the purified VHL genes were initially calculated following the formula: $OD_{260} \times 40$/molecular weight/6×10^{23}. These VHL genes with known copy numbers were serially diluted and used as templates for PCR. A standard curve was created by plotting the PCR threshold cycle of the VHL DNA with known copy numbers. The VHL copy numbers in the samples were then determined by LightCycler® software 3.5 as comparing their PCR threshold cycles to those for the standard DNA. To correct the potential differences in RNA qualities in different samples, the data were normalized by dividing copy numbers of the VHL gene by those of GAPDH.

2.4. Statistical analysis for real-time RT-PCR data

Regression analysis was conducted to predict VHL expression by age. After adjusting for age, the Student's *t*-test was used to identify the significant change of VHL expression between the exposure group and the reference control group (Fig. 3). VHL intensities were also compared between subjects with regard to skin problems by Student's *t*-test (Fig. 4) (Gladen et al., 1990).

3. Results

In this study, 29 individuals participated in the screening microarray, and 43 individuals were included in the real-time RT-

PCR study. The age distribution, alcohol use, incidence of skin problems, and PCB concentrations in the exposed versus control groups were analyzed by Student's *t*- and chi-square tests and summarized in Table 1 (Gladen et al., 1990). The serum PCB levels in the Yucheng population were far higher than those in the control group (Guo et al., 1997). The participants in the control groups were slightly older than those in Yucheng groups for both microarray and real-time RT-PCR analyses, but were similar in their status of alcohol use. A higher prevalence of chloracne and abnormal nails were observed in Yucheng subjects, consistent with our earlier findings (Guo et al., 1999).

The pooled mRNA samples by exposure status were analyzed in triplicate by microarray analysis. Expression levels (log scale) of the 908 genes were presented as a ratio to the geometric mean of the 14 housekeeping genes. Gene expression points outside of the 95% prediction interval (ellipse) of the regression line were considered different between the Yucheng and reference groups. A total of 38 of 908 genes were found outside this level (Fig. 1; supplementary Table 1).

In addition to the regression approach, hierarchical cluster analysis was performed to detect the 908 genes for their expression levels that were adjusted for those of the housekeeping genes. After log transformation of these values, the median levels of these genes were normalized and computed. The genes with a similar expression profile were classified together. We found that the genes of DNA polymerase alpha, bone morphogenetic protein 1 precursor, serine/threonine kinase 19, and VHL were down-regulated in the exposure groups (Fig. 2). The VHL gene was also found to be down-regulated in the Yucheng population as a result of the regression analysis (Fig. 1).

To verify the finding that VHL gene was indeed down-regulated in Yucheng individuals, we performed real-time RT-PCR to detect the VHL mRNA levels in Yucheng and reference subjects. In the Yucheng population, VHL gene expression was positively associated with age (Fig. 3). While adjusting for age, VHL gene expression was lower ($P = 0.04$) in Yucheng as compared to that in the control subjects (0.00017 ± 0.00002 vs. 0.00023 ± 0.00003). These findings suggested that exposure to a mixture of PCBs and PCDFs causes VHL down-regulation. Also, 11 of the 28 Yucheng study subjects have completed a questionnaire interview for medical conditions in 2003. The self-reported skin problems included chloracne, abnormal nails, and hyperkeratosis (Gladen et al., 1990). Among the 11 Yucheng subjects, those who reported having abnormal nails had lower VHL expression than those without abnormal nails (0.00012 ± 0.000053 vs. 0.00018 ± 0.000052, $P = 0.048$, Fig. 4).

4. Discussion

This is the first study to scan a large number of genes for expression in the peripheral lymphocytes of humans exposed to PCBs and PCDFs and a comparison group. Among the 908 genes scanned for stress response and DNA repair factors, tumor suppressors/oncogenes, cell cycle regulators, kinases, proteases, and transcriptional factors, we found that the VHL gene was consistently down-regulated in the exposed subjects. This was further confirmed with real-time RT-PCR. More interestingly, VHL down-regulation was associated with both the PCB exposure level and the dermatological manifestations.

Among Yucheng subjects who participated in this study, those with skin manifestations (chloracne, hyperpigmentation, and abnormal nail) had lower VHL expression compared to those without skin manifestations. Whether depression of VHL played a role in mediating such skin manifestations remain to be determined. Nevertheless, VHL mutation or down-regulation has been reported to associate with skin breakdown or psoriasis, suggesting that VHL level is associated with skin phenotypes (Stevens et al., 2004; Tovar-Castillo et al., 2007).

The exact pathway through which exposure to PCBs and PCDFs might down-regulate VHL expression is not clear, since regulation of VHL expression is not completely understood. It was found that the promoter region of the VHL gene contains a potential binding site for dioxin-responsive element (Zatyka et al., 2002), which is recognized by the ligand-bound AhR/Arnt heterodimer (Denison et al., 1989; Hankinson, 1995; Probst et al., 1993). It is possible that VHL transcription is regulated by AhR. In addition, the VHL promoter has also been found to contain the SP1 and E2F1-binding sites (Zatyka et al., 2002), indicating that its transcription is mediated through multiple transcriptional controls. Interestingly, the dioxin-responsive element contains SP1 site as well. This suggests that the gene regulation of VHL might be mediated by AhR, which is a target for some PCBs (Denison and Deal, 1990).

Besides the AhR-mediated effect, VHL expression might be affected through the hypoxia-inducible factor (HIF)-1-requiring pathway. VHL forms a stable complex with HIF-1 (Cockman et al., 2000; Ohh et al., 2000). One recent study reported that PCB exposure down-regulated HIF-1 gene expression and diminished HIF-1 function, which plays crucial role in hypoxia-induced signaling pathways (Clausen et al., 2005). HIF-1α has also been known to compete with AhR for recruitment of Arnt to form the HIF1 complex (Gradin et al., 1996). Upon PCB exposure, the preferential recruitment of Arnt to AhR is likely to cause down-regulation of HIF-1 complex, and potentially HIF-1 expression. It is likely that VHL expression is also down-regulated as a result of feedback control of HIF1 loss. Whether it is mediated through aryl hydrocarbon receptor transcription factor is not known.

VHL blocks tumor initiation, and its mutations cause cell transformation and proliferation (Lott et al., 2002). VHL is known to suppress various types of cancers (Kim and Kaelin, 2004). Loss of function of the VHL protein has been found to be associated with retinal, spinal cord, and cerebellar hemangioblastoma, renal cell carcinoma, and pheochromocytoma (Shuin et al., 2006). Hemangiomas of the adrenal gland, lung, and liver, and multiple cysts of the pancreas and kidneys are also observed with varying prevalence (Iida et al., 2004; Kaelin and Maher, 1998). Several previous studies on cancer occurrence or mortality found an increase in cancers among dioxin-exposed populations (Fingerhut et al., 1991; Flesch-Janys et al., 1998; Hooiveld et al., 1998; Ketchum and Michalek, 2005; Ott and Zober, 1996; Steenland et al., 1999). Nevertheless, in a recent mortality study of the Yucheng subjects after 24 years of follow-up, we did not see a significant increase in cancer deaths (a total of 41) among Yucheng subjects as compared to the reference population (Tsai et al., 2007). It is possible that the reduced VHL expression by PCBs/PCDFs exposure caused less dramatic carcinogenetic effects or more chronic effects than those caused by VHL gene mutation, which induces early-onset carcinogenesis. The long-term follow-up of the Yucheng subjects for future study of cancer mortality as well as cancer occurrence is warranted.

In summary, our study applied a method of screening gene expressions using a cDNA microarray system combined with subsequent statistical analysis and confirmation. Using this approach, we found reduced VHL gene expression in Yucheng men. These findings provide new insights into the potential role of VHL in disease mechanisms or biomarkers in humans exposed to PCBs and PCDFs.

Appendix A. Supporting Information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.envres.2008.07.006.

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