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Microarray analysis of gene expression in peripheral blood mononuclear cells from dioxin-exposed human subjects

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Abstract

Tetrachlorodibenzo-*p*-dioxin (TCDD) is classified as a human carcinogen and exerts toxic effects on the skin (chloracne). Effects on reproductive, immunological, and endocrine systems have also been observed in animal models. TCDD acts through the aryl hydrocarbon receptor (AhR) pathway influencing largely unknown gene networks. An industrial accident in Seveso, Italy in 1976 exposed thousands of people to substantial quantities of TCDD. Twenty years after the exposure, this study examines global gene expression in the mononuclear cells of 26 Seveso female never smokers, with similar age, alcohol consumption, use of medications, and background plasma levels of 22 dioxin congeners unrelated to the Seveso accident. Plasma dioxin levels were still elevated in the exposed subjects. We performed analyses in two different comparison groups. The first included high-exposed study subjects compared with individuals with background TCDD levels (average plasma levels 99.4 and 6.7 ppt, respectively); the second compared subjects who developed chloracne after the accident, and those who did not develop this disease. Overall, we observed a modest alteration of gene expression based on dioxin levels or on chloracne status**.** In the comparison between high levels and background levels of TCDD, four histone genes were up-regulated and modified expression of *HIST1H3H* was confirmed by real-time PCR. In the comparison between chloracne case–control subjects, five hemoglobin genes were up-regulated. Pathway analysis revealed two major networks for each comparison, involving cell proliferation, apoptosis, immunological and hematological disease, and other pathways. Further examination of the role of these genes in dioxin induced-toxicity is warranted.

Keywords: Tetrachlorodibenzo-*p*-dioxin (TCDD); Microarray; Gene expression; Biomarkers; Leukemia; Blood

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1. Introduction

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a major environmental contaminant, has a long biological half-life (≥7 years) and was listed as an established human carcinogen by the International Agency for Research on Cancer (IARC) in 1997 (IARC, 1997). TCDD exerts varying toxic effects in experimental animals, including developmental, endocrinological, immunological, and reproductive effects (Birnbaum and Tuomisto, 2000). TCDD alters the expression of a wide spectrum of genes via binding to the aryl hydrocarbon receptor (AhR) through recognition of xenobiotic response elements (XRE) (Mimura and Fujii-Kuriyama, 2003) and XRE-II (Boutros et al., 2004; Sogawa et al., 2004) in gene promoters. These genes include phase I and II xenobiotic metabolizing enzymes, as well as genes involved in cell proliferation, cell cycle regulation and apoptosis. However, downstream gene targets are largely uncharacterized and the role of gene expression changes in TCDD-associated toxicity and diseases such as chloracne and cancer are largely unknown.

In 1976, an industrial accident exposed several thousand people to substantial quantities of TCDD in Seveso, Italy (Bertazzi et al., 2001). Within several months of the accident, a large outbreak of chloracne was observed (Baccarelli et al., 2005). Follow-up of the exposed population through 1996 revealed an excess of hemopoietic neoplasms in both genders (RR 1.7, 95% CI: 1.2, 2.5) with the highest increase for non-Hodgkin's lymphoma (RR = 2.8, 95% CI: 1.1, 7.0) and myeloid leukemia (RR = 3.8, 95% CI: 1.2, 12.5), occurring after 15 years (Bertazzi et al., 2001). The increased risk of hemopoietic neoplasms in the Seveso population underlines the importance of studying long-term dioxin effects in exposed subjects.

Approximately 20 years after the exposure, high TCDD plasma levels were still present in the exposed individuals with significantly higher levels in women (Landi et al., 1997, 1998). We hypothesized that long-term presence of dioxin in these subjects could result in alteration of gene expression and that examination of global gene expression by microarray would help us to elucidate the genetic pathways involved in hemopoietic neoplasm development. We therefore chose peripheral blood mononuclear cells as a relevant target for these studies. We report here results of global gene expression analysis in a sample from the Seveso cohort comparing individuals with high levels of dioxin with those with background levels. We also compared individuals who developed chloracne immediately after the accident with those who were exposed but did not develop chloracne, to investigate gene expression changes underlying the development of chloracne.

2. Materials and methods

2.1. Study subjects

We selected 26 subjects from the Seveso cohort for this study. We obtained approvals of the institutional review boards and written informed consent from each study subject. The selection aimed at comparing subjects with extreme levels of plasma dioxin 20 years after the accident, and no major differences with regard to potential confounders. Thus, we selected only never smoker, one gender (female), and similar ages (24–49 years), alcohol consumption and medication use between groups. In addition, we previously measured plasma levels of 22 congeners in all subjects, and they did not substantially differ within this study sample. Thirteen subjects were exposed to background levels of TCDD (mean 6.7 ppt, range 4.5–7.9 ppt) and 13 exposed to high levels of TCDD (mean 99.4 ppt, range 23.9–268 ppt). The mean age was 32 and 28 years, in the high and background exposure groups, respectively. The 26 individuals could also be equally divided into two case–control groups based on development of chloracne in response to TCDD exposure. TCDD levels ranged from 74.3 to 268 ppt and from 25.2 to 90.2 ppt, in chloracne cases and subjects who did not develop chloracne, respectively. Mean age was 28 and 32 years, in cases and controls, respectively.

Based on this selection, a power calculation demonstrated that 13 matched pairs would be sufficient to have an 80% probability of selecting a true gene if the ratio of expression were 2.0 and the family-wise error rate (FWER) were controlled at 5%.

2.2. Sample collection, RNA isolation, amplification and hybridization

The increased risk of hemopoietic neoplasms in the Seveso population, in the absence of differences in the complete blood count of the exposed individuals, suggested that peripheral blood mononuclear cells (PBMC) could represent a good target for these studies. Details of sample collection and PBMC isolation have been described in (Landi et al., 2003). Briefly, PBMC were separated from whole blood (5–50 ml collected in tubes treated with sodium heparin) on a ficoll (Histopaque 1077, Sigma Chemical Co., St. Louis, MO) hypaque density gradient. The cells were washed twice and cryopreserved at a concentration of 2×10^7 cells/ml with an equal volume of freeze medium (RPMI 1640 (Life Technologies) and with 7.5% cell culture grade dimethylsulfoxide (American Type Culture Collection, Rockville, MD), 20% FBS, 2 mM glutamine, 100 U/ml penicillin and 100 μg/ml amphotericin (Life Technologies). A one ml aliquot of cells was frozen at a rate of 1 °C per minute and then stored in the vapor phase of liquid nitrogen.

Total RNA was isolated from cryopreserved mononuclear cells using RNeasy mini kits (Qiagen, Valencia, CA) according to the manufacturer's instructions and quantified using a SmartSpec™3000 (Bio-Rad, Hercules, CA).

Each of the 26 samples (100 ng) was labeled separately according to the GeneChip Eukaryotic Small Sample Target Labeling Assay Version II (http://www.affymetrix.com/support/technical/technotesmain.affx), with the exception that the GeneChip Sample Cleanup Module (Affymetrix, Santa Clara, CA) was used instead of ethanol precipitation. The protocol consists of two rounds comprising oligo-dT-primed cDNA synthesis, second-strand cDNA synthesis, and *in vitro* transcription (IVT) RNA amplification steps, with intermediate clean-up protocols after each step. During the second round of IVT biotinylated UTP is incorporated into the cRNA. The biotin-labeled RNA is hybridized to the GeneChips, washed, detected with streptavidin phycoerythrin conjugate and scanned by the GeneChip Scanner. The amount of light emitted at 570 nm is proportional to the bound target at each location on the probe array. Hybridization and scanning were performed as described in the GeneChip Expression Manual. Samples were hybridized singly to GeneChips.

2.3. Chip normalization and quality controls

Quality control indicators including initial sample quality (A260/A280 ratios between 1.7 and 2.2), integrity by gel analysis (28S ribosomal bands approximately twice the intensity of the 18S bands), amplification yields for the first and second rounds (exceeding 25 μg/ml and 1000 μg/ml, respectively) and quality of the second round amplified RNAs (A260/A280 > 1.85) were all satisfactory. Quality control metrics of the microarray raw data including noise, background, % probe sets present/absent, and 3′/5′ ratios for internal control genes (β-*actin* and *GAPDH*) were satisfactory and consistent.

To allow comparisons, all chips were scaled to a target intensity of 500 based on all probe sets on each chip. Samples were run blind so that exposure status was unknown.

2.4. Statistical analysis to identify differentially expressed genes

Robust multi-array analysis (RMA) (Irizarry et al., 2003) was used to analyze the data produced by the chips. Genes whose expression was significantly different between high- and background exposed individuals, as well as between chloracne cases versus control individuals were identified using a standard two-sample *t*-test (allowing for unequal variances in high- and low-exposed groups) and two-sided *p*-values. Expression ratios were based on the normalized mean data of all members of the group.

2.5. Pathway analysis

Gene Refseq accession numbers were imported into Ingenuity Pathway Analysis (IPA) software (Ingenuity® Systems, Redwood City, CA, http://www.ingenuity.com) a web-based application, which queries the ingenuity pathway knowledge base (IPKB) for genetic interactions. The information contained in the ingenuity pathways knowledge base is derived from the scientific literature and each connection in a network is supported by one or more publications. There exist in the literature over 139 peer-reviewed publications including IPA data, e.g. (Challen et al., 2005). Genes which can be mapped to genetic networks available in the Ingenuity database, known as "focus genes", are used to build networks and a score for each network is calculated according to the fit of the user's set of genes. Score is displayed as the negative log of the *p*-value, indicating the likelihood of the focus genes in a network being found together by random chance. Thus score of 2 have at least a 99% chance of not being generated by chance alone. In the current study a score of 10 or higher was used to select highly significant biological networks.

To evaluate the significance of the association of a particular gene set with the relevant canonical pathway within Ingenuity, a ratio of the number of genes from the data set that map to the pathway divided by the total number of genes that map to the canonical pathway is displayed and Fischer's exact test is used to calculate the corresponding *p*-value.

2.6. Quantitative real-time PCR analysis using TaqMan® gene expression assays

Total cDNA (equivalent to 20 ng input RNA), generated with the SuperScript™ First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA) was used to confirm GeneChip findings by TaqMan® Gene Expression Assays (TMGEA) (Applied Biosystems, Foster City, CA), which were run in quadruplicate under standard assay conditions. Because of its abundance and low variability between different types of lymphoid cells, the TATA box binding protein (*TBP*) was used as a normalization gene for real-time PCR. The mean baseline cycle threshold (C_t) for TATA box binding protein (*TBP;* GenBank ID NM_003194), was subtracted from the mean Ct for the other six assays to normalize results. These were then compared between high/background exposed and chloracne case/control sample groups. As PCR amplification is exponential, each cycle represents a doubling of the PCR product, so a cycle difference in relative C_t represents a two-fold difference in starting cDNA quantity. TMGEA used were TATA box binding protein (*TBP*), Hs99999910_m1; hemoglobin beta (*HBB*), Hs00758889_s1; histone 1, H3h (*HIST1H3H*), Hs00818527_s1; interleukin 8 (*IL8*), Hs00174103_m1; cytochrome p450, family 1, subfamily B, polypeptide 1 (*CYP1B1*), Hs00164383_m1; cAMP responsive element modulator (*CREM*), Hs00181804_m1; glutathione *S*-transferase M3 (*GSTM3*); Hs00168307_m1, kruppel-like factor 4 (*KLF4*), Hs00358836_m1; microsomal glutathione *S*-transferase 1 (*MGST1*), Hs00220393_m1; nuclear factor, interleukin 3 regulated (*NFIL3*), Hs00705412_s1; transducer of ERBB2, 1 (*TOB1*), Hs00271739_s1.

Table 1
Genes significantly up-regulated by dioxin exposure ranked by ratio ($N = 22$ probes/genes)

Affy ID	Ratio	p-Value	Gene title	Gene symbol	RefSeq ID
244181_at	1.497	0.0429	Phosphoinositide-3-kinase, regulatory subunit 1 (p85 alpha)	PIK3R1	NM_181504
229740_at	1.479	0.0168	PP12104	LOC643008	XM_928053
222067_x_at	1.391	0.0096	Histone 1, H2bd	HIST1H2BD	NM_021063
215528_at	1.354	0.0371	Mannosyl (alpha-1,6-)-glycoprotein	MGAT5	NM_002410
209889_at	1.350	0.0180	SEC31-like 2 (*S. cerevisiae*)	SEC31L2	NM_015490
216176_at	1.337	0.0374	Hepatocellular carcinoma-related	HCRP1	–
208527_x_at	1.326	0.0317	Histone 1, H2be	HIST1H2BE	NM_003523
224489_at	1.315	0.0179	KIAA1267	KIAA1267	NM_015443
240823_at	1.301	0.0008	*Homo sapiens*, clone IMAGE:5730164, mRNA	–	–
222139_at	1.298	0.0209	KIAA1466 gene	KIAA1466	–
215235_at	1.275	0.0465	Spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	SPTAN1	NM_003127
217152_at	1.270	0.0398	CDNA FLJ14074 fis, clone HEMBB1001869	–	–
204014_at	1.268	0.0133	Dual specificity phosphatase 4	DUSP4	NM_001394
220918_at	1.262	0.0479	Chromosome 21 open reading frame 96	C21orf96	–
208523_x_at	1.239	0.0069	Histone 1, H2bi	HIST1H2BI	NM_003525
214481_at	1.230	0.0067	Histone 1, H2am	HIST1H2AM	NM_003514
207373_at	1.230	0.0004	Homeo box D10	HOXD10	NM_002148
232613_at	1.226	0.0027	Polybromo 1	PB1	NM_018165
225929_s_at	1.216	0.0233	Chromosome 17 open reading frame 27	C17orf27	NM_020914
201853_s_at	1.210	0.0044	Cell division cycle 25B	CDC25B	NM_004358
234848_at	1.205	0.0001	Human TCRAV5.1a mRNA for T cell receptor alpha-chain.	LOC650815	XM_939906
206813_at	1.201	0.0171	Cardiotrophin 1	CTF1	NM_001330

3. Results

3.1. Differential gene expression in the high-low dioxin-exposed groups by microarray

Peripheral blood mononuclear cell RNAs from high/background exposed groups (two groups of 13 individuals each) were analyzed by Affymetrix GeneChip array. Among the top 50 most significantly altered genes (p-value ≤ 0.05) the majority of genes showed low levels (<20%) of up- or down-regulation of expression by dioxin exposure (data not shown). Aldehyde dehydrogenase 6 family, member A1 (ALDH6A1) exhibited the greatest downregulation with a ratio of 0.646 ($p = 0.0005$), while homeobox D10 (HOXD10) showed the greatest degree of up-regulation with a ratio of 1.23 ($p = 0.0004$), among the top 50 most significant genes. Given the low ratios of differential expression overall, we considered a cut-off ratio of 1.2 as having potential biological relevance. Using this cut-off, 22 genes (22 probes) were significantly up-regulated (Table 1) and 113 genes (118 probes) were down-regulated by dioxin exposure (Table 2). Five down-regulated genes *CD86*, *DNAJC10*, *IGFBP7*, *PCSK5* and *PTEN* were each identified by two different probes (Table 2). The gene with the greatest magnitude of downregulation by exposure was *HLA-DRB4* (ratio 0.30, $p = 0.0157$). The gene with the greatest degree of up-regulation by dioxin exposure was PIK3R1 (ratio 1.5, $p = 0.0429$), Table 1. Four histone genes (*HIST1H2BD*, *HIST1H2BE, HIST1H2BI* and *HIST1H2AM*) were significantly up-regulated by exposure (Table 1).

3.2. Differential gene expression in the chloracne case–control groups by microarray

Gene expression in the study population was also examined on the basis of chloracne with cases having developed chloracne after exposure and controls remaining asymptomatic for the disease. Low ratios of differential expression were observed for the top 50 chloracne-associated genes with lowest p-values (data not shown). Of note however was the fact that glutathione *S*-transferase M3 (*GSTM3*) was up-regulated as evidenced by two different probe sets (202554_s_at and 235867_at ratios 1.37 and 1.22; $p = 0.0004$ and 0.00001).

Table 2
Genes significantly downregulated by dioxin exposure ranked by ratio ($N = 118$ probes/113 genes)

Affy ID	Ratio	p-Value	Gene title	Gene symbol	RefSeq ID
209728_at	0.301	0.0157	Major histocompatibility complex, class II, DR beta 4	HLA-DRB4	NM_021983
228949_at	0.504	0.0304	G protein-coupled receptor 177	GPR177	NM_001002292
201163_s_at	0.571	0.0218	Insulin-like growth factor binding protein 7	IGFBP7	NM_001553
225207_at	0.576	0.0196	Pyruvate dehydrogenase kinase, isozyme 4	PDK4	NM_002612
209555_s_at	0.604	0.0288	CD36 molecule (thrombospondin receptor)	CD36	NM_000072
220646_s_at	0.611	0.0065	Killer cell lectin-like receptor subfamily F, member 1	KLRF1	NM_016523
230413_s_at	0.623	0.0342	Adaptor-related protein complex 1, sigma 2 subunit	AP1S2	NM_003916
226668_at	0.627	0.0011	WD repeat, sterile alpha motif and U-box domain containing 1	WDSUB1	NM_152528
227787_s_at	0.629	0.0098	Thyroid hormone receptor associated protein 6	THRAP6	NM_080651
221841_s_at	0.629	0.0361	Kruppel-like factor 4 (gut)	KLF4	NM_004235
223087_at	0.640	0.0111	Enoyl coenzyme A hydratase domain containing 1	ECHDC1	NM_018479
221589_s_at	0.646	0.0005	Aldehyde dehydrogenase 6 family, member A1	ALDH6A1	NM_005589
201218_at	0.648	0.0102	C-terminal binding protein 2	CTBP2	NM_001329
222453_at	0.650	0.0118	Cytochrome *b* reductase 1	CYBRD1	NM_024843
203574_at	0.651	0.0258	Nuclear factor, interleukin 3 regulated	NFIL3	NM_005384
238002_at	0.651	0.0311	Golgi phosphoprotein 4	GOLPH4	NM_014498
225114_at	0.662	0.0081	Alkylglycerone phosphate synthase	AGPS	NM_003659
228170_at	0.663	0.0354	Oligodendrocyte transcription factor 1	OLIG1	NM_138983
236487_at	0.664	0.0010	Hypothetical protein FLJ30655	FLJ30655	NM_144643
244187_at	0.665	0.0042	Chromosome X open reading frame 33	CXorf33	NM_198450
239346_at	0.666	0.0068	Chromosome 12 open reading frame 38	C12orf38	NM_024809
226329_s_at	0.668	0.0024	Hypothetical protein BC018453	LOC129531	NM_138798
235158_at	0.669	0.0095	Hypothetical protein FLJ14803	FLJ14803	NM_032842
223090_x_at	0.669	0.0008	Vezatin, adherens junctions transmembrane protein	VEZT	NM_017599
203973_s_at	0.674	0.0245	CCAAT/enhancer binding protein (C/EBP), delta	CEBPD	NM_005195
226665_at	0.675	0.0074	AHA1, activator of heat shock 90 kDa protein ATPase homolog 2	AHSA2	NM_152392
205922_at	0.675	0.0236	Vanin 2	VNN2	NM_004665
224436_s_at	0.676	0.0055	Nipsnap homolog 3A (*C. elegans*)	NIPSNAP3A	NM_015469
235670_at	0.677	0.0467	Syntaxin 11	STX11	NM_003764
204286_s_at	0.680	0.0196	Phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	NM_021127
203765_at	0.680	0.0284	Grancalcin, EF-hand calcium binding protein	GCA	NM_012198
226383_at	0.680	0.0030	Chromosome 11 open reading frame 46	C11orf46	NM_152316
205559_s_at	0.680	0.0059	Proprotein convertase subtilisin/kexin type 5	PCSK5	NM_006200
217427_s_at	0.681	0.0101	HIR histone cell cycle regulation defective homolog A	HIRA	NM_003325
225796_at	0.684	0.0027	PX domain containing serine/threonine kinase	PXK	NM_017771
222637_at	0.688	0.0095	COMM domain containing 10	COMMD10	NM_016144
235346_at	0.688	0.0091	FUN14 domain containing 1	FUNDC1	NM_173794
235615_at	0.690	0.0287	Protein geranylgeranyltransferase type I, beta subunit	PGGT1B	NM_005023
225367_at	0.691	0.0065	Phosphoglucomutase 2	PGM2	NM_018290

Table 2 (*Continued*)

Affy ID	Ratio	*p*-Value	Gene title	Gene symbol	RefSeq ID
223065_s_at	0.691	0.0035	STARD3 N-terminal like	STARD3NL	NM_032016
242648_at	0.692	0.0323	Kelch-like 8 (*Drosophila*)	KLHL8	NM_020803
202085_at	0.692	0.0124	Tight junction protein 2 (zona occludens 2)	TJP2	NM_004817
223423_at	0.694	0.0328	G protein-coupled receptor 160	GPR160	NM_014373
235463_s_at	0.694	0.0034	LAG1 longevity assurance homolog 6 (*S. cerevisiae*)	LASS6	NM_203463
228153_at	0.694	0.0220	IBR domain containing 2	IBRDC2	NM_182757
225769_at	0.697	0.0079	Component of oligomeric Golgi complex 6	COG6	NM_020751
226276_at	0.697	0.0336	Hypothetical protein MGC23909	MGC23909	NM_174909
222235_s_at	0.697	0.0110	Chondroitin sulfate GalNAcT-2	GALNACT-2	NM_018590
222714_s_at	0.698	0.0319	Lactamase, beta 2	LACTB2	NM_016027
204194_at	0.698	0.0236	BTB and CNC homology 1	BACH1	NM_001011545
231736_x_at	0.698	0.0311	Microsomal glutathione *S*-transferase 1	MGST1	NM_020300
227268_at	0.699	0.0018	PTD016 protein	LOC51136	NM_016125
226142_at	0.699	0.0115	GLI pathogenesis-related 1 (glioma)	GLIPR1	NM_006851
234915_s_at	0.700	0.0062	Density-regulated protein	DENR	NM_003677
238465_at	0.700	0.0065	Hypothetical protein MGC33648	MGC33648	NM_153706
204160_s_at	0.705	0.0271	Ectonucleotide pyrophosphatase/phosphodiesterase 4	ENPP4	NM_014936
228155_at	0.705	0.0039	Chromosome 10 open reading frame 58	C10orf58	NM_032333
239328_at	0.706	0.0394	RCSD domain containing 1	RCSD1	NM_052862
213222_at	0.712	0.0071	Phospholipase C, beta 1 (phosphoinositide-specific)	PLCB1	NM_015192
209686_at	0.713	0.0438	S100 calcium binding protein, beta (neural)	S100B	NM_006272
210895_s_at	0.717	0.0372	CD86 molecule	CD86	NM_006889
219147_s_at	0.718	0.0006	Chromosome 9 open reading frame 95	C9orf95	NM_017881
201888_s_at	0.719	0.0045	Interleukin 13 receptor, alpha 1	IL13RA1	NM_001560
219859_at	0.719	0.0142	C-type lectin domain family 4, member E	CLEC4E	NM_014358
211711_s_at	0.724	0.0249	Phosphatase and tensin homolog	PTEN	NM_000314
213005_s_at	0.731	0.0149	Ankyrin repeat domain 15	ANKRD15	NM_015158
204053_x_at	0.732	0.0021	Phosphatase and tensin homolog	PTEN	NM_000314
238581_at	0.734	0.0220	Guanylate binding protein 5	GBP5	NM_052942
201653_at	0.735	0.0347	Cornichon homolog (*Drosophila*)	CNIH	NM_001009551
225174_at	0.740	0.0010	DnaJ (Hsp40) homolog, subfamily C, member 10	DNAJC10	NM_018981
214084_x_at	0.741	0.0097	Similar to neutrophil cytosol factor 1 (NCF-1)	LOC648998	XM_927922
227680_at	0.742	0.0009	Zinc finger protein 326	ZNF326	NM_181781
222562_s_at	0.745	0.0267	Tankyrase	TNKS2	NM_025235
218519_at	0.748	0.0005	Solute carrier family 35, member A5	SLC35A5	NM_017945
242245_at	0.749	0.0005	Synapse defective 1, Rho GTPase, homolog 2 (*C. elegans*)	SYDE2	XM_086186
201889_at	0.749	0.0382	Family with sequence similarity 3, member C	FAM3C	NM_001040020
210176_at	0.750	0.0358	Toll-like receptor 1	TLR1	NM_003263
217955_at	0.751	0.0058	BCL2-like 13 (apoptosis facilitator)	BCL2L13	NM_015367
224967_at	0.753	0.0106	UDP-glucose ceramide glucosyltransferase	UGCG	NM_003358
201162_at	0.753	0.0451	Insulin-like growth factor binding protein 7	IGFBP7	NM_001553
202388_at	0.755	0.0301	Regulator of G-protein signalling 2, 24kDa	RGS2	NM_002923
209814_at	0.758	0.0318	Zinc finger protein 330	ZNF330	NM_014487
216652_s_at	0.759	0.0151	Down-regulator of transcription 1, TBP-binding	DR1	NM_001938

Table 2 (*Continued*)

Affy ID	Ratio	*p*-Value	Gene title	Gene symbol	RefSeq ID
226366_at	0.760	0.0203	SNF2 histone linker PHD RING helicase	SHPRH	NM_173082
204774_at	0.760	0.0112	Ecotropic viral integration site 2A	EVI2A	NM_001003927
205715_at	0.761	0.0302	Bone marrow stromal cell antigen 1	BST1	NM_004334
202539_s_at	0.762	0.0451	3-Hydroxy-3-methylglutaryl-Coenzyme A reductase	HMGCR	NM_000859
226493_at	0.762	0.0221	Potassium channel tetramerisation domain containing 18	KCTD18	NM_152387
209096_at	0.765	0.0140	Ubiquitin-conjugating enzyme E2 variant 2	UBE2V2	NM_003350
215933_s_at	0.768	0.0479	Homeobox, hematopoietically expressed	HHEX	NM_002729
229588_at	0.770	0.0123	DnaJ (Hsp40) homolog, subfamily C, member 10	DNAJC10	NM_018981
225612_s_at	0.771	0.0362	UDP-GlcNAc: betaGal beta-1,3-*N*-acetylglucosaminyltransferase 5	B3GNT5	NM_032047
205560_at	0.773	0.0008	Proprotein convertase subtilisin/kexin type 5	PCSK5	NM_006200
222752_s_at	0.773	0.0001	Chromosome 1 open reading frame 75	C1orf75	NM_018252
226283_at	0.774	0.0436	WD repeat domain 51B	WDR51B	NM_172240
201487_at	0.779	0.0312	Cathepsin C	CTSC	NM_001814
229533_x_at	0.781	0.0045	Zinc finger protein 680	ZNF680	NM_178558
202651_at	0.783	0.0187	Lysophosphatidylglycerol acyltransferase 1	LPGAT1	NM_014873
204809_at	0.783	0.0084	ClpX caseinolytic peptidase X homolog (*E. coli*)	CLPX	NM_006660
206877_at	0.785	0.0036	MAX dimerization protein 1	MXD1	NM_002357
207654_x_at	0.786	0.0158	Down-regulator of transcription 1, TBP-binding	DR1	NM_001938
201636_at	0.786	0.0362	Fragile X mental retardation, autosomal homolog 1	FXR1	NM_001013438
202026_at	0.787	0.0191	Succinate dehydrogenase complex, subunit D	SDHD	NM_003002
218398_at	0.788	0.0058	Mitochondrial ribosomal protein S30	MRPS30	NM_016640
201589_at	0.789	0.0078	SMC1 structural maintenance of chromosomes 1-like 1 (yeast)	SMC1L1	NM_006306
227322_s_at	0.790	0.0174	BRCA2 and CDKN1A interacting protein	BCCIP	NM_016567
228670_at	0.793	0.0040	Telomerase-associated protein 1	TEP1	NM_007110
204634_at	0.795	0.0154	NIMA (never in mitosis gene a)-related kinase 4	NEK4	NM_003157
236609_at	0.795	0.0096	PMS1 postmeiotic segregation increased 1 (*S. cerevisiae*)	PMS1	NM_000534
210093_s_at	0.795	0.0392	Mago-nashi homolog, proliferation-associated (*Drosophila*)	MAGOH	NM_002370
203177_x_at	0.796	0.0347	Transcription factor A, mitochondrial	TFAM	NM_003201
205686_s_at	0.797	0.0250	CD86 molecule	CD86	NM_006889
228585_at	0.798	0.0021	Ectonucleoside triphosphate diphosphohydrolase 1	ENTPD1	NM_001776
218616_at	0.798	0.0332	Integrator complex subunit 12	INTS12	NM_020395
227593_at	0.798	0.0051	Hypothetical protein LOC645580	FLJ37453	XM_928597
200977_s_at	0.799	0.0004	Tax1 (human T cell leukemia virus type I) binding protein 1	TAX1BP1	NM_006024
226962_at	0.799	0.0138	Zinc finger and BTB domain containing 41	ZBTB41	NM_194314
208127_s_at	0.799	0.0041	Suppressor of cytokine signaling 5	SOCS5	NM_014011

Table 3
Genes significantly up-regulated in chloracne cases ranked by ratio ($N = 33$ probes/23 genes)

Affy ID	Ratio	p-Value	Gene title	Gene symbol	RefSeq ID
214414_x_at	3.11	0.02158	Hemoglobin, alpha 2	HBA2	NM_000517
209116_x_at	3.04	0.0222	Hemoglobin, beta	HBB	NM_000518
211696_x_at	2.58	0.01197	Hemoglobin, beta	HBB	NM_000518
213515_x_at	2.46	0.03382	Hemoglobin, gamma A	HBG1	NM_000184
217232_x_at	2.39	0.02357	Hemoglobin, beta	HBB	NM_000518
211745_x_at	2.26	0.02166	Hemoglobin, alpha 1	HBA1	NM_000558
209458_x_at	2.06	0.02117	Hemoglobin, alpha 1	HBA1	NM_000517
217414_x_at	2.04	0.02068	Hemoglobin, alpha 1	HBA1	NM_000517
204018_x_at	1.79	0.01021	Hemoglobin, alpha 1	HBA1	NM_000517
211699_x_at	1.66	0.02107	Hemoglobin, alpha 1	HBA1	NM_000517
204848_x_at	1.60	0.02618	Hemoglobin, gamma A	HBG1	NM_000184
204419_x_at	1.59	0.04907	Hemoglobin, gamma A	HBG1	NM_000184
209967_s_at	1.55	0.02096	cAMP responsive element modulator	CREM	NM_001881
230645_at	1.49	0.01369	FERM domain containing 3	FRMD3	NM_174938
223298_s_at	1.45	0.04984	5′-Nucleotidase, cytosolic III	NT5C3	NM_001002009
209723_at	1.44	0.03159	Serpin peptidase inhibitor, clade B (ovalbumin), member 9	SERPINB9	NM_004155
230511_at	1.43	0.02038	cAMP responsive element modulator	CREM	NM_001881
202704_at	1.41	0.04464	Transducer of ERBB2, 1	TOB1	NM_005749
200864_s_at	1.40	0.00839	RAB11A, member RAS oncogene family	RAB11A	NM_004663
202554_s_at	1.37	0.00044	Glutathione *S*-transferase M3 (brain)	GSTM3	NM_000849
240456_at	1.33	0.03415	FLJ11795 protein	FLJ11795	NM_001039935
206834_at	1.33	0.03594	Hemoglobin, delta	HBD	NM_000519
204897_at	1.32	0.02418	Prostaglandin E receptor 4 (subtype EP4)	PTGER4	NM_000958
232164_s_at	1.32	0.01637	Epiplakin 1	EPPK1	NM_031308
201980_s_at	1.28	0.04271	Ras suppressor protein 1	RSU1	NM_012425
206545_at	1.27	0.00219	CD28 molecule	CD28	NM_006139
201528_at	1.27	0.02876	Replication protein A1, 70kDa	RPA1	NM_002945
201236_s_at	1.27	0.02303	BTG family, member 2	BTG2	NM_006763
230170_at	1.25	0.02054	oncostatin M	OSM	NM_020530
203725_at	1.23	0.02686	Growth arrest and DNA-damage-inducible, alpha	GADD45A	NM_001924
235867_at	1.22	0.00001	Glutathione *S*-transferase M3 (brain)	GSTM3	NM_000849
209112_at	1.22	0.03005	Cyclin-dependent kinase inhibitor 1B (p27, Kip1)	CDKN1B	NM_004064
204550_x_at	1.21	0.04847	Glutathione *S*-transferase M1	GSTM1	NM_000561

As with the exposure data in the previous section, we considered a cut-off ratio of 1.2 as having potential biological relevance and using this cut-off, 23 genes represented by 33 probes were significantly up-regulated in association with chloracne (Table 3). *CREM* was identified by two different probes. Five hemoglobin genes: *HBA2* and *HBD* (each identified by a single probe); *HBB* and *HBG1* (identified by three different probes); *HBA1* (identified by five different probes) were significantly up-regulated. As shown in Table 4, nine genes were down-regulated.

3.3. Identification of biological networks affected by dioxin exposure and chloracne status

We investigated biological interactions among the genes associated with dioxin exposure and chloracne status using the Ingenuity Pathway Analysis (IPA) tool. Analysis of the top 200 genes with the greatest magnitude of differential expression associated with dioxin exposure and chloracne, with a p-value cut-off of 0.05 and a ratio cut-off of 1.25 (up- or down-regulation), showed two significant networks each (score ≥ 10). Network genes are listed in Table 5.

3.3.1. Biological networks affected by dioxin exposure

The top-scoring network (network 1, score = 27) identified for dioxin exposure includes genes involved in cellular growth and proliferation (*KLF4*, *CD36*, C-terminal binding protein 2 (*CTBP2*)), glucose metabolism (*CD36*, pyruvate dehydrogenase kinase, isozyme 4 (*PDK4*)), and cell death (*NFIL3*, *CD36*, *CTBP2*, *KLF4*). Network 2 (score = 22, Table 5) asso-

Table 4
Genes significantly downregulated in chloracne cases ranked by ratio ($N = 9$ probes/genes)

Affy ID	Ratio	p-Value	Gene title	Gene symbol	RefSeq ID
244546_at	0.68	0.03902	Cytochrome *c*, somatic	CYCS	NM_018947
230756_at	0.74	0.03511	Zinc finger protein 683	ZNF683	NM_173574
214850_at	0.75	0.03679	Hypothetical protein LOC153561	LOC153561	NM_207331
224851_at	0.76	0.04771	Cyclin-dependent kinase 6	CDK6	NM_001259
229040_at	0.76	0.0293	Integrin, beta 2	ITGB2	NM_000211
229748_x_at	0.76	0.03728	Similar to Tektin-3	LOC389830	NM_001033515
221652_s_at	0.77	0.02217	Chromosome 12 open reading frame 11	C12orf11	NM_018164
205898_at	0.77	0.04669	Chemokine (C-X3-C motif) receptor 1	CX3CR1	NM_001337
211986_at	0.78	0.00469	AHNAK nucleoprotein (desmoyokin)	AHNAK	NM_001620

ciated with dioxin exposure includes genes involved in cell death/apoptosis (CCAAT/enhancer binding protein (C/EBP), delta (*CEBPD*), GLI pathogenesis-related 1 (glioma) (*GLIPR1*), insulin-like growth factor binding protein 7 (*IGFBP7*), *KLF4*, phosphoinositide-3-kinase, regulatory subunit 1 (*PIK3R1*), phorbol-12-myristate-13-acetate-induced protein 1 (*PMAIP1*), DNA replication, recombination, and repair (*PMAIP1*, *PIK3R1*), and cancer (*KLF4*, *CEBPD*, *IGFBP7*, *PIK3R1*).

While networks consider all possible interactions, canonical pathway analysis queries genes in pre-defined and well-characterized biological pathways. With the canonical pathway analysis, two pathways were significantly associated with dioxin exposure but differential expression of only two genes was observed in each case. In the insulin-like growth factor-1 (*IGF-1*) signaling pathway ($p = 0.0276$) insulin-like growth factor binding protein 7 (*IGFBP7*) was decreased 1.75-fold,

Table 5
High-scoring networks (score ≥ 10) identified by Ingenuity® pathway analysis

Network ID	Network	Association	Score	Focus genes	Functions
1	*AP1S2*, *CD36*, CLDN1, *COMMD10*, *CTBP2*, *DUSP4*, ELK3, *FLJ30655*, FNTA, HAS2, *HLA-DRB4*, HRAS, IL15, IL18RAP, IL1A, KLF3, *KLF4*, LIPE, MAP3K7IP2, MAPK1, *NCOR1*, *NFIL3*, NFKB1, *PCSK5*, *PDK4*, *PGGT1B*, POMC, PPARG, RLN2, SAA1, SPI1, *TJP2*, TLR4, TNFSF11, UBE2I	Dioxin exposure	27	14	Cellular growth and proliferation, carbohydrate metabolism, cell death
2	ACP1, *BACH1*, BARD1, BRCA1, CASP3, CDKN1A, *CEBPD*, CHRNA7, CRSP6, CTNNB1, DDR1, ELL, *GLIPR1*, GPS2, *HIRA*, HOXA5, *IBRDC2*, IFITM1, *IGFBP7*, IRF5, *KLF4*, PDCD8, PDE4B, *PIK3R1*, *PMAIP1*, POU4F1, PTPRF, S100A4, S100B, SAA1, SMC2L1, *SPTAN1*, *THRAP6*, TP53, *VEZATIN*	Dioxin exposure	22	12	Cell death, DNA replication, recombination, and repair, cancer
3	*AHNAK*, *BTG2*, CCND1, *CD28*, *CDK6*, CDKN2C, *CREM*, *CX3CR1*, *CYCS*, DAPK1, DMTF1, *GSTM3*, HAS1, HLA-DQA1, HLA-DQB1, HLA-DRB1, IFNG, IL6, IL8, IL17F, IL18RAP, LGALS7, LY96, PCNA, PTGER1, *PTGER4*, RFC5, *RPA1*, *RSU1*, *SERPINB9*, TGFB1, TNFRSF6B, *TOB1*, TRIM21, XCL1	Chloracne	28	13	Cell death, immunological disease, cell-to-cell signaling and interaction
4	EPO, GH1, *HBA1*, *HBA2*, *HBB*, Hbb-ar, Hbb-b1, Hbb-b2, Hbb-bh1, *HBD*, HBE1, *HBG1*, HBG2, HBQ1, HBZ	Chloracne	10	5	Hematological disease, genetic disorder, hematological system development and function

Focus genes are italicized.

while *PIK3R1* was increased 1.5-fold. *PIK3R1* along with dual specificity phosphatase 4 (*DUSP4*), which was increased by 1.27-fold, implicated the stress-activated protein kinase/c-Jun NH2-terminal kinase (*SAPK/JNK*) signaling pathway ($p = 0.031$).

3.3.2. Biological networks affected by chloracne

The top-scoring network associated with chloracne (network 3, score = 28), shown in Table 5, is related to cell death/apoptosis (BTG family, member 2 (*BTG2*), *CD28*, cAMP responsive element modulator (*CREM*), prostaglandin E receptor 4 (*PTGER*), replication protein A1, 70 kDa (*RPA1*), serpin peptidase inhibitor, clade B (ovalbumin), member 9 (*SERPINB9*), cyclin-dependent kinase 6 (*CDK6*), chemokine (C-X3-C motif) receptor 1 (*CX3CR1*), cytochrome *c*, somatic (*CYCS*)), immunological disease (*CD28* and *PTGER*), cell–cell signaling and interaction (*CD28*, *CX3CR1* and *CYCS*). The second chloracne-associated network (network 4, score = 10, Table 5) includes five hemoglobin genes shown earlier in Table 4, which are involved in hematological disease, genetic disorders and hematological development and function. All five genes were up-regulated (in some cases almost three-fold) in individuals who developed chloracne and this was confirmed for *HBB* using real-time quantitative PCR. This finding was strengthened by the fact that 13 separate probe sets identified five hemoglobin genes.

No canonical pathways were identified in the chloracne comparison.

3.4. Confirmation by quantitative real-time PCR

Ten genes were chosen for further study and confirmation by real-time PCR, based on ratio of differential expression, *p*-value, number of probe sets identifying a gene, patterns of expression (such as hemoglobin and histone families described above), and potential biological significance. Fig. 1 illustrates the correlation between expression levels measured by real-time PCR and microarray for genes associated with dioxin exposure and chloracne status, and the correspondent levels of significance are reported in Table 6. For 8 of the 10 genes chosen, real-time PCR analysis confirmed the direction of change (up- or down-regulation). *GSTM3*, which was highly significantly up-regulated in association with chloracne by microarray ($p = 0.0004$), was confirmed by real-time PCR ($p = 0.007$). *HBB* was up-regulated by high exposure ($p = 0.04$) and approached significant up-regulation in association with chloracne ($p = 0.07$) groups. Real-time PCR showed significant up-regulation of *HIST1H3H*.

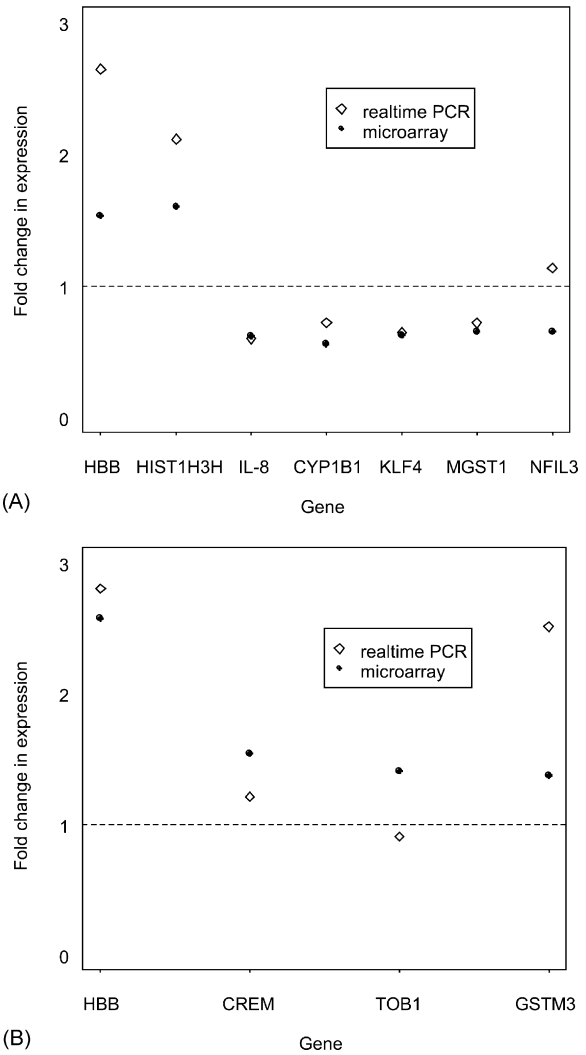

Fig. 1. Correlation of microarray and real-time PCR measurements of expression of genes associated with (A) TCDD exposure, and (B) chloracne.

4. Discussion

RNA samples obtained from 26 individuals approximately 20 years after accidental exposure to dioxin in Seveso, in 1976, were analyzed for differential gene expression associated with exposure and past chloracne status by Affymetrix GeneChip microarrays. Due to the long half-life of dioxin, current plasma TCDD levels were still substantially elevated in the exposed individuals.

Overall, we observed a modest alteration of gene expression based on dioxin levels or on chloracne status, both in terms of numbers of genes altered and expression ratios**.** In the comparison between high levels

Table 6
Expression of genes associated with TCDD exposure and chloracne by real-time PCR and microarray analyses

	Real-time PCR		Microarray	
	Fold change	*p*-Value	Fold change	*p*-Value
Genes associated with exposure				
Up				
HIST1H3H	2.11	0.03*	1.60	0.08
HBB	2.65	0.04*	1.50	0.33
Down				
IL-8	0.60	0.22	0.60	0.09
CYP1B1	0.71	0.14	0.56	0.08
KLF4	0.65	0.13	0.63	0.04*
MGST1	0.71	0.19	0.65	0.06
NFIL3	1.13	0.41	0.65	0.03*
Genes associated with chloracne				
Up				
HBB	2.81	0.07	2.68	0.02*
CREM	1.21	0.09	1.48	0.02*
GSTM3	2.52	0.007*	1.37	0.0004*
TOB1	0.91	0.53	1.4	0.04*

*Indicates significant *p*-values.

and background levels of TCDD, up-regulation of four histone genes was notable and modified expression of *HIST1H3H* was confirmed by real-time PCR. In the comparison between subjects who developed chloracne after the accident and those who did not develop the disease, up-regulation of five hemoglobin genes was noted and modified expression of *HBB* approached significance by real-time PCR. Involvement of these genes may offer new mechanistic insight into known effects of dioxin exposure on immune development and function (Kerkvliet, 2002; Luebke et al., 2006), carcinogenesis (Mandal, 2005; Nebert et al., 2000; Schwarz and Appel, 2005) and diabetes (Remillard and Bunce, 2002).

In the pathway analysis, two IPA networks associated with dioxin exposure included genes involved in cellular growth and proliferation, glucose metabolism, cell death, DNA replication, recombination and repair, and cancer. A key network gene, *KLF4*, is a member of the kruppel family of transcription factors, which mediates p53-dependent G1/S cell cycle arrest in response to DNA damage (Yoon et al., 2003) and is a potential tumor suppressor gene in colorectal cancer (Zhao et al., 2004). *KLF4* acts as a quiescence maintenance factor in B lymphocytes (Yusuf and Fruman, 2003). Reduced *KLF4* expression in our study could represent dysregulation of B cell development or, as a potential tumor suppressor gene, could be a contributory factor in TCDD-associated hematological cancers (Bertazzi et al., 2001). In the Seveso population, plasma IgG levels were decreased with increasing TCDD plasma concentration in a study conducted 20 years after the exposure, showing long-term immunologic effects of dioxin (Baccarelli et al., 2002).

Chloracne is a sensitive indicator of dioxin poisoning (Yamamoto and Tokura, 2003), but less than 0.1% of the subjects in the Seveso cohort were diagnosed with chloracne suggesting that susceptibility or environmental factors may have played a critical role (Pesatori et al., 2003) and indeed we observed chloracne-related changes in gene expression distinct from those associated with exposure. Chloracne manifests as a localized dermatological disease and dioxin toxicity in chloracne cases appeared to be confined to acute dermatotoxic effects (Baccarelli et al., 2005). However, we show changes in gene expression in PBMC of individuals with chloracne, suggesting more systemic involvement. The top-scoring network associated with chloracne is related to cell death, cell–cell signaling and interaction, and immunological alterations. In particular, a protein encoded by the *PTGER* gene (which was up-regulated in association with chloracne) is one of four receptors identified for prostaglandin E2 (PGE2). Knockout studies in mice suggest that this receptor may be involved in the initiation of skin immune responses through stimulation of T cells (Narumiya, 2003). Expression of *CD28*, whose co-stimulation is essential for CD4-positive T cell proliferation, survival, and activation (Keir and Sharpe, 2005), was also up-regulated. Indeed, a number of genes found altered in the chloracne cases are involved in the regulation of T cell activity and function, and may in turn be involved in acne-associated inflammation (Jeremy et al., 2003; Trivedi et al., 2006). The identification of several PBMC genes with potential roles in the TCDD-mediated pathogenesis of chloracne is interesting in light of a recent paper, which shows that the peripheral blood transcriptome dynamically reflects system wide biology (Liew et al., 2006).

A second chloracne-associated network included five hemoglobin genes up-regulated in individuals who developed the disease following exposure. Several studies have investigated the association between TCDD exposure and hematologic and immune functions in exposed subjects (Baccarelli et al., 2002, 2005) with inconclusive results. TCDD levels were among the highest ever reported, and yet almost all clinical laboratory tests on these individuals were normal soon after the accident; any abnormal test result, including variation of hematocrit or CBC levels, was only transitory in nature (Needham et al., 1997). We did measure hematologic and immune parameters in the subjects at the moment of this study (almost 20 years after the accident); as expected, no

significant association were found, with the exception of decreased IgG levels. These observations further suggest that the use of PBMC should not introduce a bias in the analysis of different groups. However, it is unclear what the changes in hemoglobin gene expression represent.

Expression of two members of the glutathione *S*-transferase superfamily of oxidative stress response genes was also modified, with *GSTMI* (DeJong et al., 1991) and *GSTM3* (Campbell et al., 1990) up-regulated by high TCDD in chloracne cases. These findings are in accord with previous studies in animal models, which showed a sustained oxidative stress response in mice following TCDD exposure (Shertzer et al., 1998), and another report on global gene expression (1152 genes) in waste incineration workers occupationally exposed to dioxin and PAHs (Kim et al., 2004), which showed up-regulation of five genes related to oxidative stress.

Even though the mean exposure between the chloracne case (74.3–268 ppt) and control (25.2–90.2 ppt) groups was different, each group comprised almost equal numbers of high- and low-exposed individuals. Therefore, gene expression could reflect both presence of dioxin and susceptibility to chloracne, and we cannot disentangle these effects in the present study.

The gene expression alterations described can provide important clues with regard to dioxin toxicity. However, overall there was a weak association (low ratios among the most significant genes) between gene expression and dioxin exposure or chloracne status. This may reflect an attenuated response to long-term human exposure (Steenland et al., 2004), which could not be adequately detected by microarray analysis. Moreover, even though TCDD is cleared slowly (half-life ≥ 7 years), age-related individual variations in metabolism and excretion could have occurred (Eskenazi et al., 2004), particularly in the lowest exposure group, which included slightly younger subjects. Finally, the power calculation used for study design was based on a ratio of expression of 2 or higher, while we found expression ratios below 2.0, and limitations in sample availability of suitable quality for microarray analysis precluded expanding the study. Thus, low statistical power to observe small differences may have played a role, despite selection of subjects with extreme levels of exposure, same sex, similar tobacco consumption and no differences between comparison's groups with regard to alcohol consumption, medication use or background plasma levels of 22 congeners present in the area and unrelated with the accident.

This study highlights the challenges of examining global gene expression in human exposed populations. Few studies have been reported previously and it is now clear that smaller magnitude changes in expression may be typical in these studies (Forrest et al., 2005; Wu et al., 2003). Differences in baseline expression of genes among individuals, and/or differences in genotype and metabolic activation of chemicals may underlie some of the expected variability. Among the challenges presented by these data are development of appropriate statistical analysis to discern true differential expression, and development of accurate methods to independently confirm the findings as quantitative PCR may not reliably confirm expression changes less than two-fold.

In conclusion, approximately 20 years after the accident, we have identified by microarray and confirmed by real-time PCR modest alteration in several genes potentially associated with dioxin exposure and chloracne status in a sample of subjects from the Seveso cohort. As in any such study, confirmation of the expression of these genes in a larger population is necessary to further elucidate their roles in the dioxin-induced perturbation of blood cell development, immunological response, and chloracne we have observed. Finally, our study underscores some of the technical and study design challenges inherent in these kinds of studies.

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