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Development of Exposure Biomarkers for Endocrine Disrupting Chemicals Using DNA Microarray

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DNA 마이크로어레이를 이용한 내분비장애물질 노출지표 개발

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요 약

장기간 노출 시 발암 등 인체 유해성을 갖는 환경유래 내분비장애물질(endocrine disrupting chemicals, EDCs)에 대한 선택적이고 민감한 노출지표를 개발하기 위하여 본 연구에서는 DNA microarray를 이용하였다. 피험자는 아직 특별한 질환을 갖지 않는 18세 이상 연령, 성을 맞춘 EDCs 고농도 노출군(N=16)과 저농도군(N=16)으로 구성되었다. 노출정도 구분은 10년 이상 거주지가 K산업폐기물 소각장과 2.5 km 반경 내, 외 인지에 따라 고노출군, 저노출군으로 구분하였다. 피험자의 말초혈에서 total RNA를 분리, 각 군당 3인씩 pool로 cDNA를 합성하여 oligonucleotide DNA 칩에 적용하였다. 유전자발현의 차이를 GenePixPro 4.0 software를 이용하여 분석하였다. 총 3장의 칩을 이용하여 공통적으로 저노출군보다 고노출군에서 2배 이상 발현의 증가를 보인 유전자는 plasminogen activator(PLAT) 등 12종이 관찰되었고, 1/2 이하로 발현의 감소를 보인 유전자는 kallikrein 3(KLK3) 등 29종이었다. 이 들 유전자는 PLAT 등 면역계 반응에 관여하는 유전자 및 apoptosis, transport, G protein, chromatin, 암화, 발생(development), 대사 등에 관여하는 유전자들이었다. 그러므로 KLK3 등 본 연구에서 발굴한 유전자는 향후 확대된 인구에서 본 연구 결과의 확인을 통하여 EDCs 특이적 노출지표로써, 나아가 암 등 EDCs관련 질병의 기전 및 병인학을 구명하는데 이용가치가 높다고 사료된다.

Key words : Endocrine disrupting chemicals, biomarkers, DNA microarray, exposure, waste incinerator

INTRODUCTION

Exposure to endocrine disrupting chemicals (EDCs) occurs throughout our lives by air, water, soil, food, and household products (Mori *et al.*, 2003). Dioxin is a well known EDC. Dioxin and its related compounds were produced by incomplete combustion and affect the human body to give a wide spectrum of adverse effects such as developmental, reproductive, neurobehavioral, carcinogenic, and immunological toxicities (Newbold *et al.*, 1984; Colborn *et al.*, 1996, Williams *et al.*, 2001; Newbold, 2004).

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Due to these toxic effects of EDCs, it is very important to conduct monitoring and proper risk assessments for EDCs in the environment to regulate EDCs levels.

Japanese incinerator workers who were occupationally exposed to dioxins showed induction of *CYP 1B1* (Toide *et al.*, 2003) and people who live near to waste incinerators are suspected to be high exposure to EDCs. Many researchers have tried to develop exposure biomarkers for EDCs. Recently, several biomarkers as well as cytochrome P450s (CYPs) were used for EDCs exposure (Baron *et al.*, 1998, Bouchard *et al.*, 1998; Spencer *et al.*, 1999; Toide *et al.*, 2003; Yang *et al.*, 2003). Nevertheless, it is unclear which is proper as an exposure biomarker for EDCs. Moreover, biomarkers that reflect total EDCs have not been known, because of the complexity of environmental contaminants and the lack of analytical data that document contaminant levels during critical windows of exposure up to now.

Therefore, I have researched EDCs-responsive genes using the DNA chip to develop exposure biomarkers for EDCs and studied action-mechanisms of endocrine disruptors *in vivo*.

MATERIALS AND METHODS

1. Study populations

After recruiting 38 subjects who have lived at P city with questionnaire containing age, sex, residence year, residence place, occupation, family history of cancer smoking, alcohol consumption, etc, I did my best to choose the above variations-matched subjects for the high and low EDCs-exposed groups (N = 16 for each group; male, 6; female, 10; mean age, 60 yrs). All of them have lived in the residence for 10 years or more. Study subjects were divided into 2 groups, high EDCs-exposed and low-exposed. The high EDCs-exposed group meant people who lived within 2.5 km from a waste incinerator, while the low-exposed group lived 5 to 7.5 km away from the waste incinerator.

2. RNA preparation

Three ml of peripheral blood was obtained from the each subject. Total RNA was prepared using TRIZOL LS reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and treated with RNase-free DNase I (GibcoBRL, Grand Island, NY). After electrophoresis on agar gel, the quality and the quantity of extracted RNA were measured with identification of the 18S and 28S bands.

3. cDNA microarray

Double stranded cDNA was synthesized with the Microarray cDNA synthesis kit (Roche, Mannheim, Germany) from the pooled RNA and purified with RNeasy Mini Kit (QIAGEN, Valencia, CA).

MEGAscript T7 (Ambion, Austin, TX) was used for *in vitro* transcription. During *in vitro* transcription, Cye3-UTP or Cye5-UTP was inserted into amplified experimental or control RNA. Amplified RNA was purified with RNeasy Mini Kit (QIAGEN, Valencia, CA). Purified RNA was quantified with a spectrophotometer and reprobed when the product was less then 10 μg.

Ten μg of fluorescently labeled experimental RNA was mixed with the control RNA of the same quantity. A total of 20 μg of mixed RNA was fragmented with a fragmentation buffer (4 mM Tris-acetate, pH 8.1, 10 mM K-acetate, 3 mM Mg-acetate) for 15 min at 94°C to minimize the effect induced by the cDNA length before hybridization.

Probes and targets were hybridized together for 24 hr in 3X SSC (450 mM NaCl, 45 mM sodium citrate, pH 7.0) at 42°C on the slide of Oligo-human 10K chip (Macrogen, Seoul, Korea). Hybridized slides were washed at room temperature once in 2X SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0), 0.1% sodium dodecyl sulfate (SDS) for 5 min, again in 1X SSC (150 mM NaCl, 15 mM sodium citrate, pH 7.0) for 5 min and then in 0.5X SSC (75 mM NaCl, 7.5 mM sodium citrate, pH 7.0) for 5 min.

Tree sets of pooled mRNA from different age-sex matched subjects were applied to the chip.

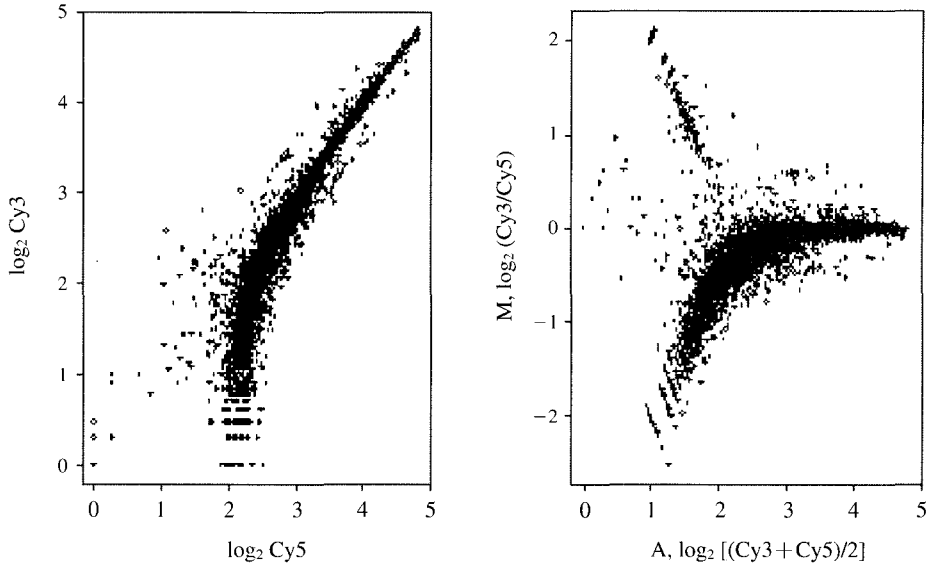

Fig. 1. Cye3/Cye5 scatter plot (left) and MA plot (right) of 10,368 genes. From the MA plot, the up- and down-regulated genes are seen in $y = \log_2|\text{Cye3/Cye5}| \geq 1$ and $x = \log_2[(\text{Cye3}+\text{cye5})/2] \geq 2$.

Table 1. Highly expressed genes in the high EDCs-exposed group compared to the low EDCs-exposed group

#	Description	Locus ID	Class	Mean of $\log_2$ (Cye3/Cye5)
1	fibroblast growth factor receptor 2, isoform 3 precursor; fgfr2	FGFR2	Immune	3.34
2	g antigen 7; gage7	GAGE1	Apoptosis	2.97
3	golgin 97; golga1	GOLGA1	Transport	2.89
4	ras-related associated with diabetes; rrad	RRAD	G protein	2.58
5	homolog of yeast mcm10; hypothetical protein pro2249; pro2249	MCM10	Chromatin/DNA	2.56
6	prostate stem cell antigen; psca	PSCA	Development	2.46
7	plasminogen activator, tissue type isoform 3 precursor; plat	PLAT	Immune	2.44
8	mhc class ii hla-dq-alpha chain; hla-dqa1	HLADQA1	Immune	2.37
9	noggin precursor; nog	NOGGIN	Cancer	2.20
10	interleukin 7 receptor; il7r	IL7R	Immune	2.19
11	cd80 antigen (cd28 antigen ligand 1, b7-1 antigen); cd80	CD80	Immune	2.14
12	thymidine kinase 2, mitochondrial; tk2	TK2	Metabolism	2.10

4. Data analysis

Cye3 and Cye5 fluorescence images were scanned using a laser confocal microscope and were analysed using a GenePixPro 4.0 program (Axon, Union, CA) to calibrate relative ratios and confidence intervals for significant determinations. The data was imported into an Excel (Microsoft) database with the corresponding gene name for analysis and normalized by S-PLUS 2000 Software (Insightful, Seattle, WA) using Lowess regression, nonlinear regression (Quackenbush, 2001).

RESULTS

Using the 3 chips, I obtained good reproducible gene expression ($p < 0.01$). From the results of the

gene expression, I found 12 genes (Table 1) that were expressed by 2 folds or higher in the high EDCs-exposed group in comparison to the low-exposed group. The 12 genes included 5 genes related to the immune system, e.g. FGFR2, PLAT, HLADQA1, IL7R, and CD80; 1 gene related to apoptosis, GAGE1; 1 gene related to transport, GOLGA1; 1 gene related to diabetes, RRAD; and others, MCM10, PSCA, NOGGIN, TK2, which are involved in chromatin, development, cancer, and metabolism, respectively.

On the other hand, I also found the 29 genes (Table 2), expression of which was 1/2 fold or less reduced in the high EDCs-exposed group compared to the low-exposed group. The 29 genes included 6 immune-related genes, e.g. IFNA6, LOC51088, CD163, IRF1, and GA17; 5 carcinogenesis-related genes, e.g. KLK3, ews protein/e1a enhancer binding protein chimera, potential tumor suppressor, OCR2, WNT 10B; and others that are involved in apoptosis, chromatin, metabolism, G protein, cell cycle, development, and transport.

From the expression results, I could conclude that long-term exposure to complex EDCs may affect immune function and carcinogenesis, etc.

Table 2. Expression- reduced genes in the high EDCs-exposed group compared to the low EDCs-exposed group

#	Description	Locus ID	Class	Mean of $\log_2$ (Cye3/Cye5)
1	b-cell receptor-associated protein bap29; bap29	BAP29	Apoptosis	−6.43
2	death receptor 6; tnfrsf21	TNFRSF21	Apoptosis	−5.49
3	swi/snf related, matrix associated, actin dependent regulator of chromatin, subfamily d, member 3; smarcd3	SMARCD3	Chromatin/DNA	−5.43
4	d-aspartate oxidase isoform a; ddo	DDO	Metabolism	−5.32
5	vasoactive intestinal peptide receptor 2; vipr2	VIPR2	G protein	−5.28
6	m-phase phosphoprotein 11; mpp11	MPP11	Cell cycle	−4.91
7	interferon, alpha 6; ifna6	IFNA6	Immune	−4.79
8	cdc6 homolog; cdc6	CDC6	Cell cycle	−4.50
9	kallikrein 3, (prostate specific antigen); klk3	KLK3	Cancer	−4.24
10	semaphorin iv; sema3f	SEMA3F	Development	−4.23
11	putative olfactory receptor; tpcr26	TPCR26	G protein	−4.15
12	golgi apparatus protein 1; glg1	GLG1	Transport	−4.00
13	ews protein/e1a enhancer binding protein chimera		Cancer	−3.64
14	excision repair cross-complementing rodent repair deficiency, complementation group 1; ercc1	ERCC1	Chromatin/DNA	−3.52
15	rev/rex activation domain binding protein-related; rab-r	RABR	G protein	−3.40
16	homolog of s. pombe mis5; mcm6	MCM6	Chromatin/DNA	−3.20
17	lymphocyte activation-associated protein; loc51088	LOC51088	Immune	−3.12
18	potential tumor suppressor		Cancer	−3.03
19	ovarian cancer-related protein 2; ocr2	OCR2	Cancer	−2.88
20	fgf2	FGF2	Development	−2.68
21	cd163 antigen; cd163	CD163	Immune	−2.62
22	interferon regulatory factor 1; irf1	IRF1	Immune	−2.45
23	dendritic cell protein; ga17	GA17	Immune	−2.39
24	cyclin e	CLNE	Cell cycle	−2.22
25	ras homolog gene family, member b; arhb	ARHB	G protein	−2.20
26	g protein-coupled receptor; gpr61	GPR61	G protein	−2.16
27	wingless-type mmtv integration site family, member 10b precursor; wnt10b	WNT10B	Cancer	−2.15
28	sphingomyelin phosphodiesterase 1, acid lysosomal (acid sphingom; smpd1	SMPD1	Apoptosis	−2.12
29	herv-h ltr-associating 2; hhla2	HHLA2	Immune	−2.11

DISCUSSION

In this study, I focused on developing the EDCs-responsive genes as exposure biomarkers for EDCs. And found that the EDCs-responsive genes were mainly related to cell cycle regulation and the immune system. The obtained 41 EDCs-responsive genes (Table 1-2) have effects on various areas of our health such as immunotoxicity, reproduction, and carcinogenicity. However, these EDCs-responsive genes are somewhat different from the already known EDCs-responsive genes that are based on *in vitro* experiments, such as pS2 (Jorgensen *et al.*, 2000) and CYP1A1 (Ishida *et al.*, 2002). Therefore, the expression changes in 41 genes in my *in vivo* study suggest that these genes can also be exposure biomarkers for environmental and mixed EDCs.

Particularly, I found high expression of PLAT in the high EDCs-exposed group in comparison to the low-exposed group. PLAT is a secreted serine protease which converts the proenzyme plasminogen to plasmin, a fibrinolytic enzyme. This enzyme plays a role in cell migration and tissue remodeling. Increased enzymatic activity causes hyperfibrinolysis which manifests as excessive bleeding. Decreased activity leads to hypofibrinolysis which can result to thrombosis or embolism. Charles *et al.* (1997) reported that 2, 3, 7, 8-tetrachlorodibenzo-*p*-dioxin (TCDD) increased mRNA levels for PLAT *in vitro*. In this study, increased expression of PLAT supports the previous *in vitro* result that PLAT can be an exposure biomarker for EDCs.

HLADQA1 is a glycoprotein, a part of the major histocompatibility (MHC) class II with beta chain. MHC class II antigen as well as class I presents peptides to T lymphocyte to activate the immune system. Karras *et al.* (1995) presented that TCDD induced upregulation of MHC antigens of the class II type and their result supports my suggestion that HLADQA1 can be a exposure biomarker for EDCs.

SMARCD3 is a member of the SWI/SNF family of proteins whose members have been thought to regulate transcription of certain genes by altering chromatin structure around those genes due to their helicase and ATPase activities. Recently, Gao *et al.* (2004) showed that a number of transcription regulators, including zinc finger proteins and SNF related proteins, are changed by benzo[a]pyrene-specific stimuli through proteomic analysis in vitro and their results support my suggestion that SMARCD3 can be a exposure biomarker for EDCs.

KLK3, a prostate specific antigen, is a subgroup of serine proteases having diverse physiological functions as well as a cancer marker highly expressed in prostatic carcinoma. Its protein product is a protease present in seminal plasma and has been thought to have normal function in the liquefaction of seminal coagulum, presumably by hydrolysis of the high molecular mass seminal vesicle protein (Lilja, 2003). Therefore, low KLK3 expression can lead to infertility. In addition to reproduction failure, low levels of KLK3 inhibit normal prostate epithelial cell proliferation reducing the size of the prostate. In addition, KLK3 has been known to be expressed throughout the androgen receptor pathway. Recently, several reports showed that AhR pathway may be related in the expression of KLK3 (Endo *et al.*, 2003): TCDD may decrease KLK3 levels by upregulation of CYPs and UDP-glucuronosyltransferase (Park *et al.*, 2004). Therefore, these reports support my results that KLK3 can be a good exposure biomarker for EDCs.

In addition, RRAD and other genes including IL7R are also considered to be candidates of exposure biomaker for EDCs.

CONCLUSION

This study suggests that the obtained epidemiology-based EDCs-responsive genes in the present study, especially PLAT, HLADQA1, SMARCD3, KLK3 and RRAD, can be exposure-biomarkers for environmental and mixed EDCs, even though, these results should be confirmed in enlarged populations.

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