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Gene Expression Profile in Iprobenfos Exposed Medaka Fish by Microarray Analysis

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Abstract

Differential gene expression profiling was carried out in the hepatic tissue of medaka fish, *Oryzias latipes*, after exposure to an organophosphorus pesticide (OPP), Iprobenfos (IBP), a widely used pesticide in agri- and fish-culture, using a medaka cDNA microarray. Twenty six kinds of differentially expressed candidate genes, with 15 and 11 induced and repressed in their gene expressions, respectively, were associated with cytoskeleton (3.8%), development (7.7%), immune (7.7%), metabolism (30.8%), nucleic acid/protein binding (42.3%) and reproduction (7.7%). Of these genes, changes at the transcription level of five were re-evaluated by real-time quantitative PCR (qRT-PCR). Considering the known function of authentic genes, the effects of IBP on the biological activity and pathological aspects in medaka fish were discussed. The identified genes could be used as molecular biomarkers for biological responses to OPPs contamination in an aquatic environment.

Keywords: *Oryzias latipes*, Organophosphoruos pesticide (OPP), Iprobenfos (IBP), Differential gene expression profile, Real-time quantitative PCR, Microarray

Organophosphorus pesticides (OPPs), because of their low environmental persistence, are most widely used in agriculture and fish farming for the control of pests, such as sea lice infestations in fish farming[1], and even in the home for stamping out noxious insects. These chemicals have high acute toxicity to non target organisms, since they are powerful inhibitors of brain cholinesterase (ChE). Thus, the studies on the effect of OPPs on organisms have mostly focused on the cholinesterase activity in various animals, including both invertebrates[2-4] and vertebrates[5]. Besides their cholinesterase inhibitory effect, OPPs are also known to induce oxidative stress[6,7], which decreases cell viability and also induce apoptosis in human NK cells[8]. However little is known about other physiological changes in aquatic organisms following the exposure to OPPs pollution within environment.

Recently, the genome wide analysis of gene expression has become possible using the cDNA microarray. Now, microarray technology is considered a powerful tool for application to various fields of biological science, such as developmental biology, physiology and toxicology, for the screening of the extensive changes in gene expression induced by certain signal(s). In addition, this technology is currently being employed to elucidate molecular mechanisms and identify potential biomarkers for specific signals, both biotic and abiotic.

The initial aim of this study was toxicogenomic screening to identify potential biomarkers for Iprobenfos (IBP, *S*-benzyl-*O*,*O*-di-isopropyl phosphorothioate) exposure in hepatic tissue of medaka fish, *Oryzias latipes*. Since IBP, an OPP, has been detected at high levels on the west coast of the Korean peninsula[9]. The second aim was to evaluate the Medaka 750 and Medaka Early-stage Embryo 2200 array (Ecogenomics Inc., Fukuoka, Japan), which contains adult 833 cDNA and embryonic 2,222 cDNA probes. As an initial identification of IBP responsive genes, this study should provide significant results for future investigation as well as the discovery of potential biomarkers for IBP exposure, toxic mechanism of IBP and; finally, the IBP mediated molecular mechanism.

Differentially Expressed Genes in IBP Exposed Medaka

To identify the genes associated with IBP induced

Table 1. Induction and repression rates of the gene expressions following IBP exposure in *O. latipes*, as obtained from the microarray analysis. The number indicates the significant difference in the fold-induction (↑) or repression (↓) from that of the control group ($P<0.01$).

Gene category	Gene title	FD*	Accession No.
Cytoskeleton	Type I cytokeratin, enveloping layer	2.20 ↑	BC065653.1
Development	Growth and differentiation factor 11	2.24 ↑	AF411599.2
	SLUG mRNA	2.00 ↓	DQ237899.1
Immune	MHC Class I Region	1.89 ↓	AB026977
	T-cell acute lymphoblastic leukemia associated antigen 1	2.17 ↓	AU178455
Metabolism	Calpain3 mRNA	2.96 ↑	AB117944.2
	Proteasome activator 28 alpha subunit (PSME1)	2.17 ↑	AF527990.1
	Globin gene cluster region	1.95 ↑	AB083077.1
	Arachidonic acid epoxygenase (cytochrome P4502J5)	1.89 ↓	AV669103
	Cytochrome P450 2D	2.08 ↓	AU180871
	Hepatic lipase	2.56 ↓	BM187526
	Adult beta-type globin	2.17 ↓	AB080120
	Embryonic alpha-type globin	3.23 ↓	AB026052
Nuc./Prot. binding	Polyglutamine binding protein variant 4 (PQBP1 gene)	2.04 ↑	AJ973596.1
	t-complex polypeptide 1 complex (TCP1)	1.99 ↑	AF164030.1
	Transposase DDE-like protein	2.03 ↑	AY864607.1
	Signal sequence receptor, gamma	2.03 ↑	BC047859.1
	Heat shock protein 27 (Hsp27)	2.01 ↑	U85501.1
	Ribosomal protein S29	1.95 ↑	BC091557.1
	XPA binding protein 1 (XAB1)	1.95 ↑	BC096466.1
	Synaptotagmin binding, cytoplasmic RNA interacting protein (SYNCRIP)	2.19 ↑	BC066570.1
	Translation initiation factor eIF-4A II	2.38 ↓	AU177129
	Hematopietic transcription factor GATA-1	1.92 ↓	AB112062
	RNA binding motif protein 8 (RBM8)	2.04 ↓	AB069905
Reproduction	Vitellogenin 1	5.06 ↑	AB064320
	Male sex-determining protein (DMRT1Y)	2.22 ↑	AY129241.1

*FD: Fold difference

toxicity, gene expression profiling in the liver of IBP exposed medaka (100 ppb, 24 h) was carried out using a medaka cDNA microarray containing about 3,055 medaka genes. From the cDNA microarray analysis, 26 reliable genes with transcript levels affected by IBP exposure were found ($P<0.01$). Of these genes, the expressions of 15 and 11 were induced and repressed, respectively (Table 1). The 26 differentially expressed genes could be categorized into 6 groups; cytoskeleton (1: 3.8%), development (2: 7.7%), immune (2: 7.7%), metabolism (8: 30.8%), nucleic acid/protein binding (11: 42.3%) and reproduction (2: 7.7%).

mRNA Quantification by Real-time Quantitative PCR (qRT-PCR)

To confirm the results of the microarray analysis and evaluate the usefulness of medaka cDNA microarry (Ecogenomics Inc., Fukuoka, Japan), qRT-PCR was carried out on five candidate genes selected from the 26 after the cDNA microarray analysis; T-cell acute lymphoblastic leukemia associated antigen 1 (TALLA1), Hepatic lipase, Heat shock protein 27 (Hsp27), RNA binding motif protein 8 (RBM8) and vitellogenin 1. The sequences of forward and reverse primers for each gene and *β-actin* are shown in Table 2. As summarized in Table 3, the changes in the gene expressions deduce by qRT-PCR in five genes, TALLA1, Hepatic lipase, Hsp27, RBM8, and Vitellogenin 1, agreed well with the result of the microarray analysis.

Discussion

The rapid rate of OPPs degradation in air and water, the limited persistence and selective toxicity have accelerated their use world-wide. However, evidence exists suggesting IBP is sufficiently persistent and concentrated in an estuary[9], which is recognized as a cardiovascular or blood toxicant, as well as a neurotoxicant. An object of this study was to estimate the potential effect of IBP, other than cholinesterase inhibition, on a fish model using a microarray analysis. From the initial screening of the differentially expressed genes after exposure to IBP in *O. latipes*, 26 differentially expressed genes, as list in Table 1, were found. qRT-PCR was also conducted to verify the results of microarray analysis. From these results, the

Table 2. The list of real-time quantitative PCR primers for the selected genes and the *β-actin* gene of *O. latipes*.

Gene		Nucleotide sequence
TALLA1	Forward	5′-GGCTGGGAAAGAAGTGTTCA-3′
	Reverse	5′-GGAGTTGGTTTGCAGGTGTA-3′
Hepatic lipase	Forward	5′-CCACATGTTCCTCCACACAG-3′
	Reverse	5′-ATCAAGAAGGTTCGCACAGG-3′
Hsp27	Forward	5′-ACGTGTCCAGGTGCTTTACC-3′
	Reverse	5′-CCTTCACGTGGAATGGTCTT-3′
RBM8	Forward	5′-CGCACTGGTGGAGTATGAGA-3′
	Reverse	5′-ATCCTGGTGTTTGCTTCCAC-3′
Vitellogenin 1	Forward	5′-AATGGACGCTTGGCCAGAAA-3′
	Reverse	5′-GCAACTGCAGGCAAGGTGAG-3′
β-actin	Forward	5′-GCCAAACCTGTACACTGACT-3′
	Reverse	5′-GAACTGCCACTTCTCATTACC-3′

Table 3. Quantification of the changes in the gene expressions of the six genes selected by real-time quantitative PCR. The microarry experimental results are compared in parallel. The number indicates significant difference in the fold-induction (↑) or repression (↓) from that in the control group.

Gene	Fold difference	
	qRT-PCR	Microarray
TALLA1	2.04 ↓	2.17 ↓
Hepatic lipase	1.44 ↓	2.56 ↓
Hsp27	2.31 ↑	2.01 ↑
RBM8	1.30 ↓	2.04 ↓
Vitellogenin 1	14.32 ↑	5.06 ↑

medaka cDNA microarry (Ecogenomics Inc., Fukuoka, Japan) was concluded to be a significantly useful for identifying differentially expressed genes in medaka fish in response to environmental pollution. Considering the known function of authentic genes, the effects of IBP on the biological activity and pathological characteristics in medaka fish will be discussed.

Growth differentiation factor 11 (Gdf11) is known to play roles in the regulation of development and differentiation. Recently, a significant higher Gdf11 transcript level has been shown in specimens obtained from colorectal cancer patient[10]. Our microarray experiment showed that the mRNA level of the Gdf11 gene was increased 2.24-fold in the IBP exposed group, suggesting IBP might induce a certain type of cancer in *O. latipes*. The SLUG gene expression has been found in migratory crest cells during the embryonic development of rats and mice, and in mesenchymal components of the lung, digestive tract, meso- and metanephros during organogenesis[11]. Recently, the SLUG gene has been characterized as a major regulator of melanocytes and melanoma cell survival. The SLUG siRNA increased cisplatin-induced cell death and was correlated with an up-regulation of the pro-apoptotic gene, PUMA[12]. In this study, the SLUG gene expression was repressed to 1/2 after exposure to IBP, potentially indicating that IBP induces cell death by enhancing the pro-apoptotic gene expression in medaka fish.

The major histocompatibility complex (MHC) is a large gene family found in most vertebrates, which is functionally involved with the adaptive and innate immune systems[13]. The IBP exposed fish showed a low MHC class 1 gene expression level compared to the control group; therefore, negative effects of IBP on the immune system of living organisms could be concluded.

T-cell acute lymphoblastic leukemia-associated antigen 1 (TALLA1) is a tetraspanin family protein normally expressed in neurons, and certain vascular endothelial and epithelial cells, but not in any hematopoietic cells[14]. Accordingly, the ectopic expression of TALLA1 has been shown to be closely related to leukemogenesis. From both our microarray experiment and qRT-PCR results, the mRNA expression of TALLA1 was down-regulated 2.17- and 2.04-fold, respectively in the IBP exposed fish group. The biological function of TALLA1 remains to be studied intensively; therefore, the effects of a low TALLA1 transcript level can not be estimated at this time.

The calcium-dependent protease, calpain, is known to have various biological functions, including apoptosis, cell division and myogenic differentiation. Recent data suggest that Calpain 3 may protect cells against apoptosis[15]. The amount of Calpain 3 mRNA was increased 2.96-fold in the IBP exposed group. From this result, it may be implied there is an increased necessity to protect cells following IBP exposure. Proteasome activator 28 (PA28) activates the hydrolysis of small non-ubiquitinated peptide using 20 S proteasome. PA28 binds to proteasome; thereby, stimulating its activity. PA28 has two homologous subunits, PA28α and PA28β, with the carboxyl terminus of α subunit being necessary for PA28 binding to proteasome as well as proteasome activation[16]. In the present study, the expression of the medaka homolog of the PA28α gene was found to be up-regulated 2.17-fold in the IBP exposed fish group. This might indicate that IBP exposure leads to proteasome activation through an increase in the PA28α transcript level. Arachidonic acid, a main component of brain and nerve cells, is transformed to various metabolites by numerous enzymes. Arachidonic acid epoxygenase (cytochrome P450 2J5, CYPIIJ5), one of the enzymes involved in arachidonic acid metabolism, catalyzes the metabolic pathway from arachidonic acid

to epoxyeicosatrienoic acids (EETs) and diepoxyeichosadienoic acids (DEEDs)[17]. EETs are known to have a cerebral arterioles dilatation function. The microarray experiment in our study showed that the transcript level of CYPIIJ5 from the IBP exposed group decreased 1.89-fold compared to the unexposed control group, suggesting cerebral apoplexy might occur due to IBP exposure. Cytochrome P450 (CYP1A) is responsible for the metabolism of many xenobiotic compounds, pesticides and petroleum products. Induction of CYP1A mRNA by organic pollutants has been widely reported in various kinds of fish[18-21]. Conversely, repression of CYP1A (CYP1A1 and CYP1A2) transcription due to oxidative stress has also been observed in hepatocytes[22]. In the microarray experiment, Cytochrome P450 2D was detected, with its expression found to be repressed by IBP exposure.

Hepatic lipase (HL), a lipolytic enzyme synthesized by hepatocytes, is localized at the surface of liver sinusoid capillaries[23]. Recent investigations have shown that 17β-estradiol (E2) represses the HL gene transcription[24]. IBP was also suggested to have endocrine disrupting ability, since the HL gene expression level was down-regulated 2.56-fold in the IBP exposed group in our microarray experiment. The result of the qRT-PCR also showed a 1.44 fold decrease in the amount of the HL gene transcript.

Polyglutamine binding protein 1 (PQBP1) is primarily present in neurons. The late-onset of the motor neuron disease-like phenotype has been observed in transgenic mice over-expressing the human PQBP1 gene[25]. The PQBP1 gene expression level was found to be increased about 2-fold in the IBP exposed group, indicating IBP exposure might lead to neuronal dysfunction. The t-complex polypeptide 1 complex (TCP1) has a chaperonin function, a critical role in the cytoskeleton, such as tubulin and actin folding[26]. The transcript level of TCP1 in IBP exposed fish was increased 1.99-fold. Considering the known function of TCP1, IBP exposure can affect the biogenesis of the cytoskeleton.

Heat shock protein 27 (Hsp27) has been shown functions on thermotolerance, cytoprotection and support cell survival under stress conditions as a chaperone, and is also involved in the apoptotic signaling pathway[27]. The transcript level of the medaka Hsp27 homologous gene was increased 2.01-fold following IBP exposure. The results of the qRT-PCR also revealed that IBP exposure induced Hsp27 transcription (2.31-fold). Thus, IBP exposure could be expected to induce cell apoptosis in fish species.

The xeroderma pigmentosum group A (XPA) has a function in nucleotide excision repair, and XPA binding protein 1 (XAB1) interacts with XPA. XAB1 seemed to have GTPase activity, since it has the motif conserved in the GTP-binding protein[28]. In this study, the XAB1 gene expression was induced about 2-fold following IBP exposure, indicating that IBP might increase nucleotide breakage. Synaptotagmin binding cytoplasmic RNA interacting protein (SYNCRIP, or heterogeneous nuclear ribonuclear protein Q1/NSAP1) plays roles as a component of mRNA granules in the neurons, and is suggested to be important for the stabilization of mRNA[29]. A higher level (2.19-fold) of the SYNCRIP transcript was observed in the microarray analysis in the IBP exposed fish compared to the control group. GATA-1 is a transcription factor essential for erythroid cell development, which is expressed mainly in the hematopoietic system[30]. From our results, GATA-1 gene transcription was shown to be down-regulated 1.92-fold following IBP exposure; thus, IBP might have a negative effect on hematopoiesis. RNA binding motif proteins (RBM) play key roles in the posttranscriptional regulation of gene expressions in eukaryotic cells, and are known to interact with a candidate tumor suppressor[31]. From both the microarray experimental and qRT-PCR results, the RBM8 gene expression level was down-regulated 2.04- and 1.30-fold, respectively, in the IBP exposed medaka compared to the control group, suggesting IBP exposed fish might have a high chance of tumorigenesis.

Vitellogenin (Vg) is an egg yolk precursor protein only expressed in female fish, but is dormant in male fish under normal conditions. Vg has been widely introduced in ecological toxicology as a powerful biomarker for feminization of male fish, which is induced by endocrine disrupting chemicals (EDCs). The transcription of Vg gene was induced 5.06- and 14.2-fold by IBP exposure in the microarray and qRT-PCR, respectively. These results suggest the high possibility of endocrine disrupting effects of IBP to biological organisms. The double sex and *mab-3*-related transcription factor 1 (DMRT1) is a first vertebrate master sex-determining gene and a putative transcription factor probably involved in testes formation in different vertebrate lineages. In medaka fish, the DMRT1 gene functions as a male sex-determining gene[32].

Methods

Animals, Exposure to Chemical

Six month old medaka fish, *O. latipes*, the d-2R strain were obtained from the Korea Institute of Toxicology (Daejeon, Korea). Fish were fasted for 2 days, and then exposed to IBP (ChemService, USA)

dissolved in dimethyl sulfoxide (DMSO) (100 ppb) for 24 hr. After rendering the animals unconscious by cold shock, the liver was excised and total RNA extracted. Three individuals were assigned to an experimental group, with their pooled RNAs used for the experiment. A group exposed to 0.001% DMSO was used as the control group.

Total RNA Extraction and Preparation of cDNA Probe

An RNeasy Mini Kit (QIAGEN, Inc, Valencia, CA, USA) was used for the extraction and purification of the medaka hepatic total RNA, with 1 μg each of the extracted total RNA reverse-transcribed with the T7-oligo dT primer to synthesize single-stranded cDNA. Subsequently, double-stranded cDNA was synthesized from the ss-cDNA, and then *in vitro* transcribed with amino-allyl UTP to generate amino-allyl labeled aRNA target samples. The aRNA samples were purified, coupled with amine reactive fluorescent dye, Cy5, with the Cy5-coupled aRNA samples then repurified for hybridization on the medaka cDNA microarray. All the processes from reverse-transcribing the total RNA to Cy5-aRNA synthesis were carried out with an Amino Allyl MessageAmp aRNA kit (Ambion Inc, Austin, TX, USA).

cDNA Microarray and Gene Expression Analysis

The cDNA microarray used in this study was fabricated by Ecogenomics, Inc. (Fukuoka, Japan), which contained 833 adult and 2,222 embryonic medaka cDNA gene probes. The detailed information of these gene probes are listed at the following web sites:

http://www.ecogenomics.co.jp/Medaka750_GeneFunction_Sept2006.pdf;

http://www.ecogenomics.co.jp/Ol_Egg_EGArray_2222GeneList.pdf.

Each of the labeled target samples were hybridized on two cDNA microarrays. Hybridizations of the labeled target samples and the gene probes on the microarray were performed for 16 hours at 42°C in 45 μL of 50% formamide (Wako, Osaka, Japan)/5 × SSC (SIGMA, St. Louis, MO)/0.5% SDS (Ambion Inc, Austin, TX) hybridization solution in a moisture chamber, followed by post-hybridization washing (two washes in 1 × SSC/0.2% SDS at 42°C for 5 minutes and 15 minutes, two washes in 0.1 × SSC /0.2% SDS at ambient temperature for 5 minutes each, then final two washes in 0.1 × SSC at ambient temperature for 2 minutes each). The process-completed microarray slides were scanned using a GenePix 4000B scanner (Molecular Devices, Sunnyvale, CA, USA) with a resolution of 10 μm.

Statistical Analysis

For the statistical analysis of the microarray data, the raw data collected by the GenePix 4000B scanner were independently normalized by the median expression value for each of the microarray, and then integrated into the ArrayStat z-test (Imaging Research Inc, St. Catharines, ON, Canada), with a significance determination of $P<0.01$, to obtain the differential gene expression ratio (also expressed "fold difference" or "FD") for each of the gene probes on the microarray. The FD values were calculated by taking the ratio between the average of six signal strengths (3 spots × 2 microarrays) for the control group and the average of six signal strengths (3 spots × 2 microarrays) for the IBP exposed experimental group.

mRNA Quantification by Real-time Quantitative PCR

The template cDNA samples for this analysis were prepared by reverse transcribing each of the exposed and unexposed medaka liver total RNA samples with SuperScript II RNase H- Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA) and Anchored Oligo $(dT)_{23}$ Primers (Sigma-Aldrich, St. Louis, MO, USA). The qRT-PCR was set up in triplicate for each gene per sample using the SYBR Green Realtime PCR Master Mix (TOYOBO, Osaka, Japan), and then processed in a 7300 Real Time PCR System (Applied Biosystems, Foster City, CA, USA).

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