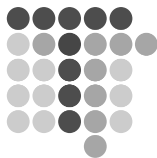

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Differentially Expressed Genes in Marine Medaka Fish (*Oryzias javanicus*) Exposed to Cadmium

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Abstract

To screen the differentially expressed genes in cadmuim-exposed marine medaka fish (*Oryzias javanicus*), a candidate marine test fish for ecological toxicity, the differential display polymerase chain reaction (DD-PCR) was carried out, since the genome-wide gene expression data are not available in this fish species yet. A total of 35 clones were isolated from cadmium-exposed fish and their nucleotide sequences were analyzed. The differentially expressed gene candidates were categorized to response to stimulus (3); ion binding (3); DNA binding (1); protein binding (6); carbohydrate binding (1); metabolic process (4); biological regulation (3); cellular process (2); protein synthesis (2); catalytic activity (2); sense of sight (1); immune (1); neurohormone (1); signaling activity (1); electron carrier activity (1) and others (3). For real-time quantitative RT-PCR, we selected catalase, glucose-6-phosphate dehydrogenase, heat shock protein 70, and metallothionein and confirmed that cadmium exposure enhanced induction of these four genes.

Keywords: *Oryzias javanicus*, Cadmium, Differential gene expression, Biomarker, Environmental stress

The exploration of genes for which expression changes with exposure to ecotoxicants or pollutants can provide important information about the reaction mechanisms in the body as well as adaptations to exterior stimuli or environmental changes. Such genes also can be developed as biomarkers to detect environmental pollution. In marine ecosystems, anthropogenic contamination, such as sewage containing persistent organic pollutants and a variety of toxic chemicals from land runoff, is one form of environmental stress. Stress can affect living organisms in many ways and can manifest itself on a variety of levels of organization. The stress caused by anthropogenic chemical substances released into the environment can be observed at very early stages of exposure on the sub-organismal level. In this study, we focused on the molecular level and screened sub-cellular level biomarkers for effects of exposure to heavy metals and for the development of tolerance and resistance.

Understanding the effects of toxicants on marine organisms has important implications. DNA damage induced early in an organism's life inhibits development to adulthood, which in turn might affect the food chain and/or cause serious economic loss in fisheries. In addition, the extinction of a species in marine ecosystem would affect its community. Therefore, detecting the biological effects of pollutants on marine organisms under low levels of contamination as well as monitoring the health status of marine ecosystems is necessary for protection of marine biological resources.

The species belong to the genus *Oryzias* have great potential as model animal for questions on biological phenomena (*O. latipes*[1]) and test fish for environmental changes (*O. latipes*[2]; *O. sinensis*[3]). The Javanese medaka (*Oryzias javanicus*), which is distributed in marine environments around the Indonesian Islands and the Malay Peninsula, is capable of surviving in freshwater[4,5]. According to Inoue and Takei, *O. javanicus* adults had a 100% survival rate after direct transfer from seawater to a 50% seawater/freshwater mixture, and no difficulties in spawning and egg hatching were apparent. Like the Japanese medaka (*O. latipes*), which is a frequently used labora-

Table 1. Differentially expressed genes identified by DD-PCR in Cd-exposed *Oryzias javanicus*.

Clones	Size (bp)	Putative identity[a]	Accession No.[b]	DD-PCR[c]
		Response to stimulus		
Oja027	209	Metallothionein	AY906860	Up
Oja029	393	Selenium-dependent glutathione peroxidase	EF692639	Up
Oja036	267	Catalase	AY734528	Up
Oja073	421	Warm-temperature-acclimation-related-65 kDa-protein-like-protein	AB075199	Up
		Ion binding		
Oja017	442	NAD(P)H dehydrogenase quinone 1	DQ387060	Up
Oja048	213	Alcohol dehydrogenase 1 (ADH1)	AY917130	Up
Oja071	222	Ceruloplasmin	AF336125	Up
		DNA binding		
Oja067	203	Polyglutamine binding protein variant 4 (PQBP1 gene)	AJ973596	Up
		Protein binding		
Oja025	309	cathepsin L (catl2 gene)	AJ872184	Up
Oja062	605	Suberites domuncula mRNA for sorcin (sorc gene)	AM040448	Up
Oja064	344	Neuregulin 2 (Nrg2)	XM_001053475	Up
Oja065	290	Myosin, heavy chain 10, non-muscle (MYH10)	NM_205474	Up
Oja074	352	Serum amyloid A protein	AF300706	Up
Oja078	302	Complement component C3	AB021653	Up
		Carbohydrate binding		
Oja049	411	Cellulose-binding elicitor lectin (CBEL)	DQ849335	Up
		Metabolic process		
Oja055	209	*Oryzias latipes* gene for membrane guanylyl cyclase OlGC1	AB021490	Up
Oja056	214	Pyruvate dehydrogenase (pdh gene)	AM263445	Up
Oja058	522	Glucose-6-phosphate dehydrogenase	AB111384	Up
Oja072	280	Fructose-1,6-bisphosphate aldolase	X82278	Up
		Biological regulation		
Oja054	206	*Oryzias latipes* SLC21A11 gene for solute carrier family 21 member 11	AB092650	Up
Oja066	541	NTAK alpha 2	D89996	Up
Oja079	520	Transferrin	AJ300650	Up
		Cellular process		
Oja028	420	Glycine max beta-carotene hydroxylase	AY575953	Up
Oja030	395	Heat shock protein 70	D13669	Up
		Protein synthesis		
Oja007	660	Elongation factor 1 alpha (EF1-alpha)	EF555089	Down
Oja010	409	*Hippocampus comes* 40S ribosomal protein S29	AY357068	Up
		Catalytic activity		
Oja059	324	Citrate synthase (CS gene)	AM263442	Up
Oja075	322	Betaine-homocysteine methyltransferase	BC13515	Down
		Sense of sight		
Oja001	251	partial cryaA gene for alphaA-crystallin, exons 1-2	AJ617734	Down
		Immune		
Oja004	280	TLR9 gene for Toll-like receptor 9	AB234024	Down
		Neurohormone		
Oja011	346	Allatotropin neuropeptide precursor	AJ566903	Up
		Signaling activity		
Oja016	574	Epidermal growth factor (EGF)-like precursor (egfl gene)	AM231310	Up
		Electron carrier activity		
Oja034	423	Cytochrome P450-related ptotein	EF451959	Down
		Others		
Oja043	541	Mucoepidermoid carcinoma translocated protein	BC126489	Up
Oja050	622	*Oryzias latipes* hox gene cluster	AB232919	Up

[a]Basic Local Alignment Search (BlastX) at NCBI
[b]Nucleotide sequence showing the highest similarity
[c]Up- or down-regulated in Cd-exposed marine medaka (24 hr)

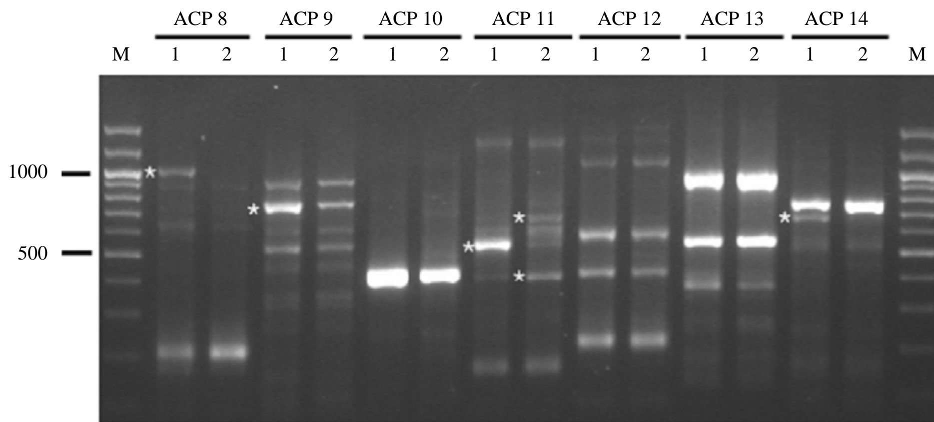

Figure 1. Differential display of mRNA from *Oryzias javanicus* exposed to cadmium (Cd) (primer set 8-14). ACP: annealing control primer; M: DNA ladder marker; 1: non-exposed control fish; 2: Cd-exposed fish. Asterisks indicate the differentially expressed genes.

tory animal, *O. javanicus* is small in size, easy to culture, frequently spawns transparent eggs, and has a short life span. Thus, the seawater- and freshwater-adaptable Javanese species may represent a new and useful species for environmental risk assessment in aquatic environments[6-10].

Differential display polymerase chain reaction (DD -PCR) is a useful tool for hunting for genes with expressions that are up- or down-regulated by exterior stimuli. In DD-PCR, mRNA expressed by a cell population are reverse transcribed and then amplified by many separate polymerase chain reactions[11]. Arbitrary PCR primers and conditions are chosen so that any given reaction yields a limited number of amplified cDNA fragments, permitting their visualization following gel electrophoresis. This relatively simple procedure allows identification of genes that are differentially expressed in different cell populations[12].

In this study, we hunted for cadmium (Cd)-responsive genes in *O. javanicus* using DD-PCR to excavate genetic biomarkers for ecotoxicity assessment of harmful chemicals. Among candidate genes, we also investigated molecular-level changes in gene expression in fish affected by exposure to cadmium by real-time quantitative PCR (qRT-PCR).

Differentially Expressed Genes in Cd Exposed Marine Medaka

As the result of initial screening, we obtained 35 amplified fragments whose expressions altered after exposed to Cd and compared them with fragments from non-exposed control fish using the DD-PCR technique (Table 1). The nucleotide sequence of each clone was analyzed by Basic Local Alignment Search (BlastX) at NCBI and categorized as their expected cellular functions. Of the 35 clones, 3 were categorized as response to stimulus, 3 as ion binding, 1 as DNA binding, 6 as protein binding, 1 as carbohydrate binding, 4 as metabolic process, 3 as biological regulation, 2 as cellular process, 2 as protein synthesis, 2 as catalytic activity, 1 as sense of sight, 1 as immune, 1 as neurohormone; 1 as signaling activity, 1 as electron carrier activity and 3 were not categorized specifically.

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

To reconfirm the gene expression changes obtained by DD-PCR, real-time quantitative PCR was performed on four candidate genes. For qRT-PCR, two groups of fishes were exposed to 10 and 100 ppb Cd for 24 hours, respectively. We selected metallothionein (Oja027) and catalase (Oja036) from response to stimulus category; glucose-6-phosphate dehydrogenase (Oja058) from carbohydrate metabolism category; and heat shock protein 70 (Oja030) from cellular process category. As showed in Figure 2, the changes in the gene expressions deduce by qRT-PCR in all four genes, agreed well with the result of the DD-PCR.

Discussion

DD-PCR was developed to identify and isolate differentially expressed genes and has been applied extensively to a range of differential gene expression analyses because of its effectiveness and convenience. One of the merits of this technique is that it requires only a small amount of RNA. However, a major handicap of this technique is its relatively high chance of yielding a false-positive. Many efforts have been made to improve the specificity of DD-PCR[13]. Recently, an epochal modification to eliminate false-positives by increasing the annealing specificity with a specially designed annealing control primer (ACP) system has developed[14]. In this study, we applied the ACP system to identify the differentially expressed

genes that responded to Cd; Figure 1 shows the agarose gel image of the DD-PCR products. The GeneFishing kit (a commercial brand based on the ACP system) that we used has two merits, which derive from the high specificity of primers. First, PCR products could be separated on agarose gel because of the improved specificity and sensitivity result in only a few amplified products. Second, non-radioactive detection of the products is convenient. We previously used this system to screen differentially expressed genes in diesel oil-exposed rockfish (*Sebastes schlegeli*) and found that oil contamination induced up- or down-regulated gene expression of the obtained clones[15]. The goals of this study were to discover genes for which transcription changed with exposure to an environmental toxicant, especially Cd to develop a simple and efficient system, such as a cDNA chip, that can be used to assess contamination at ecotoxicant-impacted sites.

Clone Oja027, metallothionein (MT) is ubiquitous low molecular mass proteins (typically 6-7 kDa), rich in cysteines (about 30% of the total residues) and heavy metals (7-10 equivalents per mol, depending on the metal). They are thought to play a variety of functions, including homeostasis of the essential oligo-elements zinc and copper, defense against the harmful effects of toxic metals like cadmium and mercury, and protection from stress conditions[16]. In our previous report, we confirmed MT gene expression changes by exposure to various heavy metals and its up-regulation by Cd-exposure[8]. In this study, we also found a differentially expressed MT gene fragment using DD-PCR and confirmed its transcriptional changes in 10 ppb (1.3-fold) and 100 ppb Cd-exposed fish (2.4-fold) (Figure 2).

Clone Oja030, whose level of expression was up-regulated after Cd-exposure, was very similar to the mRNA for heat shock protein 70 cognate of *O. latipes* (DDBJ/EMBL/GenBank accession no. D13669). We submitted our nucleotide sequence to the DDBJ/EMBL/GenBank nucleotide sequence database as *O. javanicus* heat shock protein gene (DQ660324). The heat shock protein family plays an important role in a variety of physiological processes, including protein chaperoning and general cellular stress responses, and has been proposed as a biomarker of environmental stress, such as toxicant exposure[17,18]. The increased gene expressions of heat shock proteins in carp (*Cyprinus carpio*) exposed to elevated temperature and Cd treatment was reported[19], and the metal-acclimated trout showed an increase in the transcription of HSP70 compared with a reference population[20].

In this study, the expression level of the Oja030 clone increased by 1.3 fold in 10 ppb and 1.6 fold in 100 ppb Cd-exposed *O. javanicus*, as shown by real-time RT-PCR.

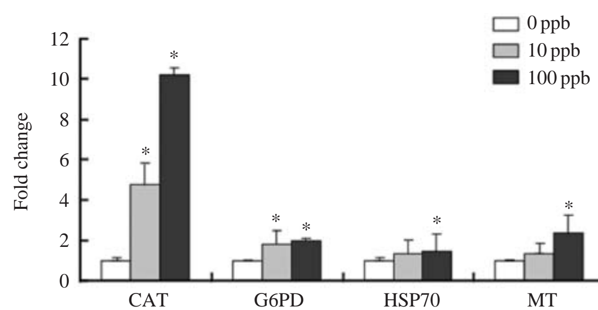

Figure 2. Expression changes of CAT, G6PD, HSP70 and MT induced by Cd-exposure. Fish was exposed to 0, 10 and 100 ppb Cd for 24 hr and RNAs were extracted from livers. The mRNA levels of CAT, G6PD, HSP70 and MT gene were evaluated by real-time quantitative RT-PCR and expressed relative to β-actin expression levels. Each histogram represents the fold-change relative to control $\pm$S.D. *Significantly different from each unexposed group ($P < 0.05$).

Clone Oja036 was homologous to *Oplegnathus fasciatus* catalase (CAT) mRNA (GenBank accession No. AY734528), and we submitted the nucleotide sequence as *O. javanicus* catalase gene (DQ660330). Catalase has the primary function of splitting hydrogen peroxide into water and oxygen; in most animal species, it generally is used as a marker enzyme[21]. According to our real-time RT-PCR results, CAT gene expression in *O. javanicus* exposed to Cd increased approximately 5-fold at 10 ppb and by 10-fold at 100 ppb in the liver (Figure 2). Up-regulation of the CAT gene in *O. javanicus* exposed to Cd corresponded to other results from fish[22], mice[23], a nematode (*Caenorhabditis elegans*)[24], and mussels (*Mytilus edulis*)[25] exposed to heavy metals.

A fourth clone, Oja058, that showed high expression after 24 hr Cd-exposure, was homologous to *O. latipes* G6PD mRNA for glucose-6-phosphate 1-dehydrogenase (G6PD) (GenBank accession No. AB1113 84). We submitted the nucleotide sequence of the isolated fragment as *O. javanicus* glucose-6-phosphate dehydrogenase gene (DQ660331). Using real-time quantitative RT-PCR, we reconfirmed the results of the DD-PCR and Cd treatment resulted in 1.8-fold (10 ppb) and 2-fold (100 ppb) enhanced induction of G6PD in liver. The oxidative stress caused by heavy metal exposure leads to the use of antioxidant defenses, and some relationship between oxidative stress markers and antioxidant enzyme activities has been well reported[22,26-28]. Research into the direct effect of Cd on G6PD gene expression in fish is rare, but in-

creased G6PD mRNA level and activity caused by heavy metal exposure have been reported for PC12 cells[29]. Increased hepatic G6PD activity induced by Cd exposure was reported in Nile tilapia (*Oreochromis niloticus*)[30], and an increased activity in brown mussels (*Perna perna*) induced by exposure to other toxic heavy metals was observed[31].

In conclusion, we successfully isolated genes that are differentially expressed in response to exposure to cadmium and confirmed the expression changes of several genes using real-time quantitative RT-PCR. The obtained clones are related to pathogenesis, extrinsic stresses, the immune system, and catalytic metabolites. Considering the expected functions of the isolated genes, the ACP-based DD-PCR method might be strong and effective way to isolate the differentially expressed genes and provide knowledge about gene expression relative to heavy metal impact. Furthermore, our results might be useful for developing biomarkers to assess marine environmental stress or heavy metal contamination.

Materials & Methods

Animals, Cd Exposure and RNA Extraction

Marine medaka (12-14 months, 3.0-3.5 cm, male) were maintained as described[8]. Two groups of five male fishes were transfer to 2 L beaker containing 1.5 L of seawater. After acclimation for 48 hrs, one group was assigned to an experimental group which exposed to 100 ppb Cd (Kanto Chemical Co. Inc., Tokyo, Japan) for 24 hr and the other group to a non-exposed control group. After rendering animals unconscious with cold shock, we excised the livers and extracted total RNAs from control and exposed groups; RNA from all controls was pooled, as was RNA from the exposed individuals. Trizol Reagent (Invitrogen, Carlsbad, CA, USA) was used for RNA purification according to the manufacturer's instructions.

First Strand cDNA Preparation and Differential Display (DD-) PCR

The first strand cDNA was synthesized using moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, Madison, WI, USA) following the manufacturer's directions. The total RNA was used as a template and primed with oligo $(dT)_{15}$-ACP (Seegene, Seoul, Korea). DD-PCR was performed using the GeneFishing DEG kit (Seegene, Seoul, Korea) to identify differentially expressed genes. We followed the manufacturer's directions for ACP (annealing control primer)-based GeneFishing-PCR. One hundred twenty arbitrary ACP primers (A1-A120) were applied to the PCR. The PCR products were separated in 2% agarose gel. The differentially expressed PCR products were cut out from the gel and extracted using the QIAquick gel extraction kit (Qiagen, Germany). The purified PCR products were cloned into the pGEM-T Easy vector (Promega). We sequenced positive clones using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Forster City, CA, USA).

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

To reconfirm the changes in gene expression induced by Cd exposure, expression changes of the four clones-catalase (DDBJ/EMBL/GenBank accession no. DQ660330, forward 5′-GTT CAA GGT GTC TCC AGA TGT GGC-3′; reverse 5′-GTC TGA ACC CTG TTT CCA TAG TCT-3′), glucose-6-phosphate dehydrogenase (DDBJ/EMBL/GenBank accession no. DQ660331, forward 5′-GGC AAG AAG GAC TCC AAG TT-3′; reverse 5′-GCC CAC ACA CAC CAG CTT CA-3′), heat shock protein 70 (DDBJ/EMBL/GenBank accession no. DQ660324, forward 5′-CAA TGA GGT CAT CAG CTG GTT G-3′; reverse 5′-TCA ATG GTT GGT CCA GAT GAT C-3′), and metallothionein (DDBJ/EMBL/GenBank accession no. AY906860, forward 5′-GGA ACC TGC AAC TGC GGT GGA TCC-3′; reverse 5′-GTC TTC CCT TTG CAC ACA CAG CCA-3′)-in 10 and 100 ppb Cd-exposed fish were quantified using real-time quantitative RT-PCR analysis. This was performed in triplicate in 384-well plates with an Applied Biosystems Prism 7900 Sequence Detection System; the β-actin gene (DDBJ/EMBL/GenBank accession no. DQ660329, forward 5′-GAT CTG GCA TCA CAC CTT CTA CAA-3′; reverse 5′-TAC ATG GCA GGG GTG TTG AAG GTC-3′) was used as an internal control. The nucleotide sequences of amplified target gene fragments from real-time RT-PCR also were reconfirmed (data not shown). The thermal conditions for PCR were: 40 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. Each real-time quantitative RT-PCR proceeded with serially diluted cDNA (1×, 0.5×, and 0.25×), which was used to generate relative standard curves for the β-actin and target genes.

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