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Medaka DNA Microarray: A Tool for Evaluating Physiological Impacts of Various Toxicants

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Abstract—A variety of bioassays and biomarkers have been developed to evaluate the effects of chemicals on the survival, development, growth, reproduction and physiology of various organisms. Development of toxicogenomics in the mid-1990s, facilitated by the advent of DNA microarray technologies, remarkably expands the scope and depth of toxicological perspective by assessing the effects of chemicals on the expression of virtually all known genes simultaneously within a single microarray. In this study, gene profiling of Japanese medaka, *Oryzias latipes*, exposed to various chemicals (heavy metals, organic solvents, pesticides, etc.) was performed using oligonucleotide-based DNA microarray representing 26,689 TIGR *Oryzias latipes* Gene Indeces. The microarray analyses identified signature gene subsets indicative of changes in physiology following exposure to various chemicals. The genes identified could serve as novel biomarkers for lesions on tissues caused by toxic substances. Furthermore, assessment of distance and similarity between gene expression measures was also attempted in order to estimate the degree of physiological changes under different conditions. Results demonstrate the possibility of using the gene expression profiling to estimate or predict the overall physiological outcomes of organisms exposed to various chemicals. (This work was supported by Japanese Science and Technology Agency (JST)).

Keywords: medaka, DNA microarray, Euclidean distance, Pearson correlation coefficient

INTRODUCTION

Environmental pollutants are discharged into our environment and can cause toxic effects on a variety of organisms either directly or indirectly through chemical/biochemical reactions. Numerous studies have been conducted to

evaluate the toxic effects of pollutants on the survival, development, growth, reproduction and physiology of various organisms using a variety of bioassays (OECD, 1992; USEPA, 1993). Molecular and biochemical techniques, called biomarkers, have also been used to elucidate toxic mechanisms. Moreover, a newly developed research field, toxicogenomics, the use of comprehensive gene expression analyses to evaluate multiple types of toxicity, greatly expands the scope and depth of toxicological approach (Irwin *et al*., 2004; Snape *et al*., 2004). Toxicogenomic studies have been remarkably facilitated by the development of the DNA microarray technology. DNA microarray technology burst onto the scene of molecular biological research in the mid-1990s (Ramsay, 1998; Schena *et al*., 1998), and promises to revolutionize biological research and further our understanding of biological processes. The advent of DNA microarrays has provided a means for analyzing the expression of thousands or even tens of thousands of genes simultaneously. This advantage allows toxicologists to take a global perspective on toxicity, in that the effects of a chemical on the expression of virtually all known genes can be evaluated within a single microarray (Ramsay, 1998).

Japanese medaka (*Oryzias latipes*), one of the major species for eco-toxicological study, is widely accepted as an experimental vertebrate model for development, histology and sexual differentiation research (Yamamoto, 1975; Ozato *et al*., 1992; Matsuda *et al*., 2002; Schartl, 2004). Large-scale gene expression analyses have also been applied to medaka developmental biology (Katogi *et al*., 2004; Kimura *et al*., 2004). Furthermore, a medaka draft genome has recently been published (Kasahara *et al*., 2007). These experimental resources promise to greatly facilitate toxicogenomic research on medaka as an ideal vertebrate animal model for sexual differentiation and development.

In this study, we assessed changes in mRNA expression caused by various toxicants with an oligonucleotide-based medaka DNA microarray developed by NimbleGen Systems, Inc. (Madison, WI, USA) using the Maskless Array Synthesizer (MAS) (Singh-Gasson *et al*., 1999). We first determined 96 hours median lethal concentrations (96 hr-LC50s) for 11 toxicants. Mature medaka were exposed to the toxicants at the 96 hr-LC50s and further subjected to DNA microarray analyses. Changes in the mRNA expression profile of the chemical-exposed fish were compared with the normal patterns of gene expression in male medaka in order to identify the genes responsible for toxic effects caused by the toxicants. Furthermore, degrees of physiological changes in medaka were also calculated based on both similarity of the mRNA expression profiles and intensities of mRNA expression levels (Kishi *et al*., 2006, 2007). Results suggest that the physiological impacts of toxicants can be estimated using the DNA microarray data.

MATERIALS AND METHODS

Test organism

Japanese medaka, *Oryzias latipes* (orange-red variety or "Himedaka"), were

originally obtained from National Institute of Environmental Studies (Tsukuba, Ibaraki, Japan) and then maintained at Japan Pulp & Paper Research Institute, Inc. for several generations. The brood stock was maintained at 24 ± 1°C in UV-disinfected, de-chlorinated, carbon-treated tap water with a 16h light-8h dark photoperiod. The fish were fed *Artemia nauplii* (<24 h after hatching) twice daily. Medaka selected for this study were approximately 6 months post-hatch and fully mature (body wt ~400 mg; total length ~3 cm). Growth conditions followed guidelines recommended by the international toxicity test protocol (OECD, 1992; http://www.env.go.jp/chemi/kagaku/).

Exposure conditions

Toxicants used in this study were $CuSO_4$, $CdCl_2$, tributyltin (TBT), zinc pyrithione (Zn–Pt), methanol (MeOH), ethanol (EtOH), dimethyl sulfoxide (DMSO), sodium lauryl sulfate (sodium dodecyl sulfate: SDS), Roundup (RU), thiuram (TMTD), and formaldehyde (FAD). Ninety-six-hours median lethal concentrations (96 hr-LC50s) for these chemicals were determined according to the standard fish acute toxicity test procedure (OECD, 1992; http://www.env.go.jp/chemi/kagaku/). Seven mature female and male medaka were separately exposed to the toxicants for four days in 3-litre glass beakers containing two litres test solutions at the 96 hr-LC50s. The test solution in each beaker was renewed daily with UV-disinfected, de-chlorinated, carbon-treated tap water. Throughout the exposure period, the DO concentration (mean ± SD) was 8.1 ± 0.1mg/l, and pH was 7.5 ± 0.1. The water temperature in all test chambers was 24 ± 0.6°C. On the last day of exposure, fish that survived in each treatment group were flash-frozen in liquid nitrogen and stored at –80°C for RNA extraction.

Construction of medaka microarray

60-mer oligonucleotide probes for each TIGR (The Institute for Genomic Research) *Oryzias latipes* (Japanese medaka) Gene Index (OLGI) were designed based on the data from OLGI, updated May 17th, 2004 (release 5.0) (Kishi *et al*., 2007). The total number of OLGIs was 26,689, including 12,849 TCs (Tentative Consensus), 13,669 singleton ESTs (Expressed Sequence Tag), and 171 singleton ETs (Expressed Transcript), with seven probes for each OLGI. The medaka microarray was synthesized using the MAS Technology (NimbleGen Systems, Inc., Madison, WI, USA) (Singh-Gasson *et al*., 1999).

Microarray analysis

Total RNA from whole frozen medaka was isolated using Qiagen RNeasy Lipid Tissue Midi Kit (Qiagen, Inc., Valencia, CA, USA) following procedures recommended by the manufacturer (Kishi *et al*., 2007). Total RNA from whole frozen medaka was first converted to double-stranded cDNA, followed by the synthesis of biotin-labeled cRNA using in vitro transcription as described elsewhere (Eberwine *et al*., 1992; Nuwaysir *et al*., 2002). cRNA was then purified and fragmented to an average size of 50 to 200 bp. Hybridizations were performed

Table 1. Ninety six hours median lethal concentrations (96 hr-LC50s) of medaka for toxic chemicals.

Chemicals*	Unit	96 Hr-LC50s			No. of tests
		Average	Max.	Min.	
$CuSO_4$	μg/l	499	580	435	9
Cd	mg/l	7.69	8.27	7.34	4
MeOH	% (v/v)	2.48	2.86	1.77	6
EtOH	% (v/v)	1.50	1.74	1.05	6
DMSO	% (v/v)	3.53	3.61	3.43	3
Formaldehyde	mg/l	35.9	39.9	25.9	4
SDS	mg/l	34.4	35.5	33.8	3
TBT	μg/l	23.6	26.0	21.6	4
Zinc pyrithione	μg/l	218	260	196	4
Thiuram	μg/l	600	650	540	4
Round up	mg/l	77.9	79.0	76.0	4

*MeOH, methanol; EtOH, ethanol; DMSO, dimethyl sulfoxide; SDS, sodium lauryl (dodecyl) sulfate; TBT, tributyltin.

with cRNA derived from single biosource (one-color hybridization). Hybridization, washing and scanning were carried out following standard procedures (NimbleGen Systems, Inc.). The expression level of each OLGI was calculated by averaging the intensities of signals from seven different probes. The signals between each array were normalized using RMA (Robust Multi-chip Analysis) normalization (Irizarry *et al.*, 2003a, b, c). GeneSpring 7.1 (Agilent Technologies, CA, USA) was used for further expression analysis.

Calculation of degrees of physiological changes in the exposed medaka

Euclidean distances and Pearson correlation coefficients (Pearson CC) (Knudsen, 2002) between gene expression data were in order to estimate degrees of physiological changes (Kishi *et al.*, 2006, 2007).

The Euclidean distance of a series of expression data $x = \{x_1, x_2, ..., x_n\}$ from origin is defined as

$$d = \sqrt{\sum_{i=1}^{n} \left(x_i - \underline{x}\right)^2}$$

where $\underline{x}$ is the average values in x (in this case, the means of 26,689 OLGIs in the controls or chemicals-treated groups).

The Pearson CC between two series of expression data $x = \{x_1, x_2, ..., x_n\}$ and $y = \{y_1, y_2, ..., y_n\}$ is defined as

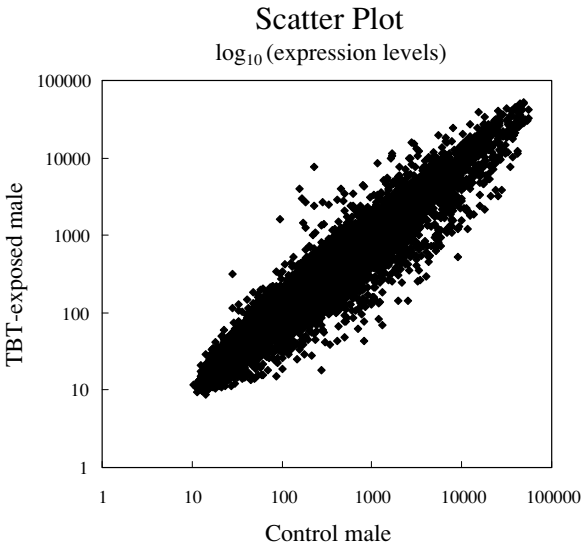

Fig. 1. A scatter plot of medaka microarray analyses of mRNA expression in control male vs. TBT-exposed male.

$$\text{Pearson CC} = \cos\theta = \frac{\sum_{i=1}^{n}\left(x_i - \underline{x}\right)\left(y_i - \underline{y}\right)}{\sqrt{\sum_{i=1}^{n}\left(x_i - \underline{x}\right)^2}\sqrt{\sum_{i=1}^{n}\left(y_i - \underline{y}\right)^2}}$$

where θ is a vector angle between the two data sets, and $\underline{x}$, $\underline{y}$ is the average values in x, y, respectively (in this case, the means of 26,689 OLGIs in the controls or chemical-treated groups). The Pearson CC can range between –1 and 1, with 1 indicating complete identity between the two series, 0 indicating no correlation, and –1 indicating negative correlations between all loci. Euclidean distance preserves the information about the magnitude of changes in the mRNA expression levels directly, whereas the Pearson CC reflects the similarity in expression patterns (Knudsen, 2002). Thus, the Euclidean distances of the expression data sets were calculated first, and then the Pearson CC values were combined with the Euclidean distances in order to estimate the degree of physiological impacts caused by the toxicants.

RESULTS AND DISCUSSION

Effects of toxicants on gene expression profiles of medaka

The 96 hr-LC50 values for toxicants used in this study are shown in Table 1.

Table 2. Concentrations for chemical exposures and numbers of significantly induced and suppressed OLGIs.

Chemicals*	Conc.	$p < 0.05$, ratio >2.0 or <0.5		
		Male	Female	Both
$CuSO_4$	0.5 mg/l	382	551	87
Cd	7.69 mg/l	591	464	153
MeOH	2.5% (v/v)	345	511	146
EtOH	1.5% (v/v)	593	429	124
DMSO	3.5% (v/v)	736	274	143
Formaldehyde	36 mg/l	368	591	204
SDS	35 mg/l	131	305	35
TBT	0.0236 mg/l	831	527	238
Zinc pyrithione	0.218 mg/l	186	275	50
Thiuram	0.6 mg/l	717	454	184
Round up	77.9 mg/l	1367	302	110

*MeOH, methanol; EtOH, ethanol; DMSO, dimethyl sulfoxide; SDS, sodium lauryl (dodecyl) sulfate; TBT, tributyltin.

Mature male and female medaka were thereafter exposed to the toxicants for 4 days at the 96 hr-LC50s for further DNA microarray analysis.

We previously confirmed that variances between mRNA expression profiles in control medaka were small (Kishi *et al.*, 2006, 2007), implying that highly reproducible physiological data can be obtained from medaka maintained under the standard conditions (OECD, 1992). In other words, the high reproducibility of physiological state of controls makes medaka an ideal model animal for evaluating physiological changes caused by various chemicals. In contrast to the high correlation between the control-control comparisons, gene expression profiles of the chemical-exposed medaka were significantly different from those of controls (Fig. 1), indicating that exposure to the toxicants at the 96 hr-LC50s strongly affected gene expressions of medaka.

In comparisons between the gene expression levels of control medaka and medaka exposed to each chemical, OLGIs which showed statistically significant differences between controls and the exposed-medaka were first selected by an analysis of variance (ANOVA) with Student's *T*-test (*p*-value cutoff 0.05). Among these selected OLGIs, changes in averaged expression levels more than two-fold and less than 0.5-fold were then considered to indicate induction and suppression, respectively. Table 2 summarizes the numbers of induced and suppressed OLGIs in medaka treated with the toxicants.

Degrees of physiological activities in the chemical-exposed medaka

Although the induced/suppressed gene sequences were determined for the toxicant exposures, only little information about toxic mechanisms can be obtained from a list of induced/suppressed gene sequences. This is because most

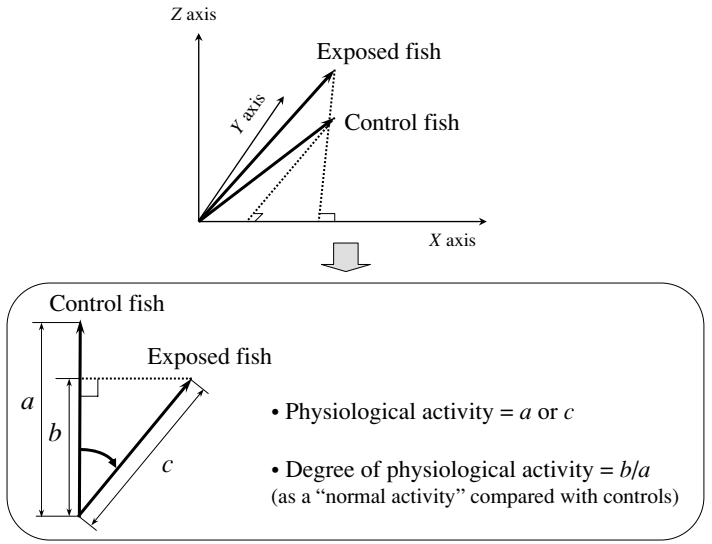

Fig. 2. Calculation of degrees of physiological activities in exposed fish. Physiological activities and degrees of physiological activities were defined as described in the text.

of the OLGIs have not yet been assigned or characterized for functions. It has also been argued that induction/suppression of biomarker gene expression such as vitellogenin may not necessarily cause physiological impairment to test animals (Yokota *et al*., 2001; Kang *et al*., 2002). Thus, it is critical to develop a method to utilize such massive gene expression data so that DNA microarray analysis can be a revolutionary tool in toxicological research field.

Because changes in the patterns of gene expression within a living cell can represent changes in the physiological state, analysis of changes in gene expression profiles should allow the evaluation of physiological changes caused by multiple types of toxicity even if many OLGIs are not known for functions. Thus, we attempted to estimate degrees of damage on physiological activities by using the entire gene expression profiles obtained for the toxicant-exposed medaka. Such an analysis cannot easily be done by pair-wise comparisons between the expression levels of several biomarker gene sequences.

We employed Euclidean distance and Pearson correlation coefficient (Pearson CC) (Knudsen, 2002) to calculate similarity/distance between gene expression measures. Using the combination of those two equations, degrees of impacts were calculated for the toxicant-exposed medaka. Figure 2 shows a model diagram of the spatial relationship between control fish and exposed fish. The expression patterns of selected genes in each group are represented as two different vectors. The directions of the vectors might be varied because of differences in gene expression patterns. Physiological activities of fish were estimated as the lengths of the vectors, "a" and "c" in the diagram. A degree of physiological activity was

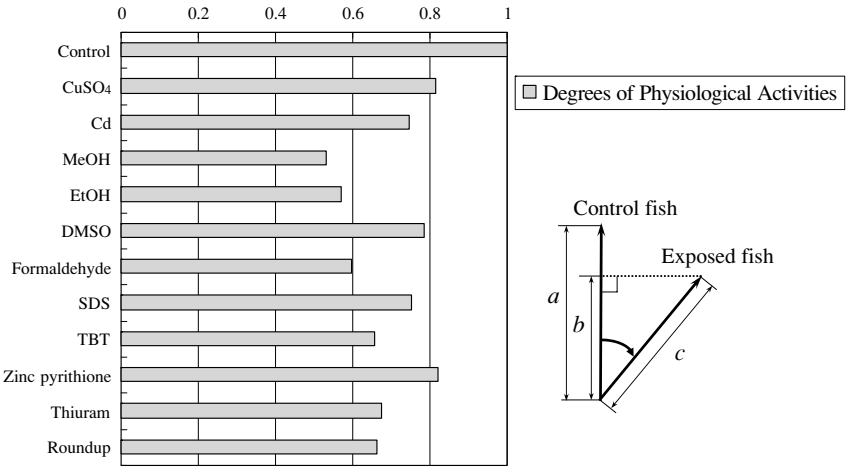

Fig. 3. Degrees of physiological activities for toxicant-exposed medaka. All numbers shown are calculated when the degree of physiological activity for control is defined as 1.0.

defined as a physiological activity of the exposed fish "as a normal fish", "b" divided by "a" in the diagram.

To determine the degree of physiological activities for the toxicant-exposures, we selected 4,155 OLGIs, which were considered as induced or suppressed for either male or female in at least one chemical treatment group (Table 2). With the 4,155 OLGIs, degrees of physiological activities were calculated for each chemical-exposure group. Figure 3 shows the average values of degrees of physiological activities for six individuals consisted of three males and females. Results clearly indicate that physiological activities were suppressed by all of the toxicant exposures at the 96 hr-LC50s, even though some OLGIs were induced or stimulated by those toxicants examined in this study (data not shown). Although similar exposure conditions, 96 hr-LC50s, were used for the toxicants, the degrees varied considerably among the toxicant-exposure groups, suggesting that contributing factors to these degrees should be different between the toxicant treatments.

Effects of toxicants on molecular functions of medaka

For further evaluating the physiological effects by the toxicants, OLGIs were first categorized into some groups based on the function or identification of the gene product in the TIGR gene ontology (GO) database version 5.0. One of the major categories in the TIGR GO database is "Molecular Function". Sub-categories and numbers of entries in the Molecular Function are listed in Table 3. The Molecular Function category includes important physiological activities such as catalytic activity, antioxidant activity, and some regulator activities, even though the number of entries in this category (2,099 gene sequences) is only less

Table 3. Numbers of entries in Molecular Function sub-categories of TIGR Gene Ontology database version 5.0.

Sub-categories*	No. of entries
Binding	1306
Catalytic activity	857
Transporter activity	355
Structural molecule activity	324
Transcriptional regulator activity	213
Signal transducer activity	198
Enzyme regulator activity	109
Chaperone activity	84
Translation regulator activity	69
Antioxidant activity	23
Total	2099

*"Obsolete molecular function", "Molecular function unknown", and "Motor activity" in Molecular Function sub-categories in TIGR Gene Ontology database are omitted in this table.

than 10% of total OLGIs (26,689 gene sequences).

Degrees of physiological activities were calculated for gene sequences listed in each sub-category (Table 4). Among the sub-categories, five groups (Binding, Catalytic activity, Transporter activity, Structural molecule activity, and Transcriptional regulator activity) were mostly suppressed by the chemical exposures. Particularly, Catalytic activity sub-category was severely damaged in some of the toxicant exposures, such as MeOH, EtOH, and FAD. On the other hand, other five groups (Signal transducer activity, Enzyme regulator activity, Chaperone activity, Translation regulator activity, and Antioxidant activity) were stimulated in some degree by the chemical exposures, except MeOH, EtOH and FAD exposures. For instance, $CuSO_4$ stimulated Signal transducer activity and Antioxidant activity sub-categories, whereas DMSO stimulated Enzyme regulator activity and Translation regulator activity sub-categories. Interestingly, MeOH, EtOH and FAD appeared not to stimulate any molecular functions significantly. These calculated degrees clearly indicate which function was damaged or stimulated by the chemical exposures, suggesting that the DNA microarray experiments and the calculations used in this study could also be applied for evaluating toxic mode of action on physiological functions.

In this study, we demonstrated that physiological impacts of organisms exposed to various toxicants could be estimated using same methodology. Degrees of chemical-dependent inhibition or stimulation of particular physiological functions could also be determined, suggesting that toxic mode of action can be evaluated by analyzing which physiological function is inhibited or stimulated. Previously, we also estimated degrees of feminization and male-dysfunction (feminization and male-dysfunction factors, respectively) on the exposure to estrogenic chemicals, using the combination of the DNA microarray experiments

Table 4. Degrees of physiological activities calculated for OLGIs in Molecular Function sub-categories of TIGR Gene Ontology database version 5.0.

Chemicals*	Binding	Catalytic activity	Transporter activity	Structural molecule activity	Transcriptional regulator activity	Signal transducer activity	Enzyme regulator activity	Chaperone activity	Translation regulator activity	Antioxidant activity
Control	1	1	1	1	1	1	1	1	1	1
$CuSO_4$	0.93	0.87	1.03	0.95	0.77	1.35	0.94	1.00	1.02	1.51
Cd	0.93	0.76	0.83	0.95	0.94	1.11	1.30	1.06	1.16	1.10
MeOH	0.69	0.54	0.78	0.73	0.73	0.87	1.04	0.95	0.95	0.94
EtOH	0.73	0.52	0.93	0.84	0.81	1.01	0.95	1.07	0.92	0.98
DMSO	0.88	0.77	0.92	0.88	0.97	1.12	1.52	1.09	1.27	1.02
Formaldehyde	0.74	0.50	0.74	0.82	0.79	0.88	1.09	0.94	1.04	0.99
SDS	0.85	0.71	0.88	0.91	0.88	1.00	1.14	1.09	1.08	1.07
TBT	0.83	0.61	0.81	0.92	0.92	1.05	1.19	1.24	1.16	1.19
Zinc pyrithione	0.89	0.76	0.96	0.97	0.88	0.93	1.14	1.10	1.06	0.96
Thiuram	0.86	0.80	0.90	0.92	0.90	1.07	1.21	1.00	1.16	1.08
Round up	0.84	0.68	0.87	0.88	0.84	0.99	1.18	1.02	1.08	0.90

*MeOH, methanol; EtOH, ethanol; DMSO, dimethyl sulfoxide; SDS, sodium lauryl (dodecyl) sulfate; TBT, tributyltin.

and same calculations (Kishi *et al*., 2006, 2007). The calculated feminization and male-dysfunction factors were in line with biological observations and histological analyses. Taken together, we propose that the combination of distance/similarity calculations and gene expression profiling could be used to estimate overall effects of multiple types of toxicity, because both the magnitude of changes in the mRNA expression levels and changes in the expression patterns are taken into account. We are now hoping that we will be able to estimate or predict environmental risks of various chemicals by using the methodology described in this study.

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