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Toxicogenomic analysis of immune system-related genes in Japanese flounder (*Paralichthys olivaceus*) exposed to heavy oil

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Abstract

Heavy oil contamination is one of the most important environmental issues. Toxicities of polycyclic aromatic hydrocarbons (PAHs), including immune toxicities, are well characterized, however, the immune toxic effects of heavy oil, as a complex mixture of PAHs, have not been investigated. In the present study, we selected Japanese flounder (*Paralichthys olivaceus*) as a model organism, and observed alteration of immune function by the exposure to heavy oil. To analyze the expression profiles of immune system-related genes, we selected 309 cDNAs from our flounder EST library, and spotted them on a glass slide. Using this cDNA array, alteration of gene expression profiles was analyzed in the kidneys of flounders exposed to heavy oil. Six Japanese flounders (mean body weight: 197 g) were acclimated to laboratory conditions at 19–20 °C. Three fish were exposed to heavy oil C (bunker C) at a concentration of 3.8 g/L for 3 days, and the others were kept in seawater without heavy oil and used as the control. After the exposure period, the fish were transferred into control seawater and maintained for 4 days, and then they were dissected and their kidneys were removed. Total RNA was extracted from the kidney samples to use in gene expression analyses. The microarray detected alteration of immune system-related genes in the kidneys of heavy oil-exposed flounders, including down-regulation of immunoglobulin light chain, CD45, major histocompatibility complex class II antigens and macrophage colony-stimulating factor precursor, and up-regulation of interleukin-8 and lysozyme. These results suggest that pathogen resistance may be weakened in heavy oil-exposed fish, causing a subsequent bacterial infection, and then proinflammatory genes may be induced as a defensive response against the infection. Additionally, we found candidate genes for use as biomarkers of heavy oil exposure, such as N-myc downstream regulated gene 1 and heat shock cognate 71 kDa proteins.

Keywords: Heavy oil; Japanese flounder; Immune system; Toxicogenomics

1. Introduction

Oil spills have occasionally occurred as a consequence of marine accidents, such as the shipwrecks of oil tankers (e.g. the *Nakhodka* in Japan in 1997 and the *Prestige* in Spain in 2002). Dispersed oil severely affects the ecosystem as well as fish farming around the accident area. Dispersed crude oil leads to the induction of xenobiotic metabolizing enzymes such as cytochrome P4501A and the formation of DNA adducts in fish (Stagg, 1998; Stagg et al., 1998; Aas et al., 2000). Polycyclic aromatic hydrocarbons (PAHs), the major components of crude oil, are known to cause these effects. The immune toxicities of PAHs are also well known (reviewed by Reynaud and Deschaux, 2006), but the toxic mechanisms of PAHs have not been fully characterized. Moreover, the immunotoxic effects of heavy oil, as a complex mixture of PAHs, have not been investigated.

Microarray technology has been applied to toxicological research, and is becoming a standard tool to investigate the molecular changes caused by toxic substances. In fish species, several research groups have constructed microarray

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platforms to monitor exposure to environmental contaminants (Denslow et al., 2007), such as Japanese medaka (*Oryzias latipes*) exposed to 17β-estrardiol (Kishi et al., 2006), zebrafish (*Danio rerio*) to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (Handley-Goldstone et al., 2005), rainbow trout (*Oncorhynchus mykiss*) to carbon tetrachloride and pyrene (Krasnov et al., 2005), and European flounder (*Platichthys flesus*) to cadmium (Williams et al., 2006). Moreover, microarray methods also have been used to identify the genes responding to a mixture of metals *in vitro* (Bae et al., 2002) or a mixture of hydrocarbons in mussels (*Mytilus* spp.) (Dondero et al., 2006). Thus, global gene expression analysis using microarrays is one of the best approaches to elucidate the mechanisms of toxic substances and to predict their effects.

The Japanese flounder (*Paralichthys olivaceus*) is an important fisheries resource in Japan, and this species has been used in toxicological research (Shimasaki et al., 2003) and in environmental monitoring (Oshima et al., 2004). Additionally, the immune system of this species has been well characterized (Kurobe et al., 2005). Therefore, in the present study, we selected *P. olivaceus* as a model species and constructed a cDNA microarray spotted with 309 immune system-related genes. Alteration of gene expression profiles was monitored in the kidneys of flounders exposed to heavy oil in order to determine the effects of the oil on immune function.

2. Materials and methods

2.1. Heavy oil exposure

Sexually immature Japanese flounders (mean body weight: 197 g) were kindly provided by Ehime Prefectural Chuyo Fisheries Experimental Station (Iyo, Japan). Fish were acclimated in 125-L tanks equipped with a filtering system in our laboratory. Rearing seawater was aerated and was kept at 19–20 °C. For the exposure test, we prepared two 13-L glass aquaria filled with 8-L seawater, and transferred three fish into each tank. One group was exposed to heavy oil (Bunker C) at a concentration of 3.8 g/L for 3 days, and the others were kept in seawater without heavy oil and used as the control. During the exposure period, the fish were not fed, and the water was aerated but not changed. After the exposure, the oil-exposed fish were transferred into another aquarium filled with control seawater and maintained for 4 days.

2.2. Microarray experiment

The flounder (*P. olivaceus*) EST library was established by Digital Genomics (Seoul, Korea). All sequences were subjected to BLASTX homology search against a non-redundant protein database using the BLASTALL program. To construct a cDNA array, 309 selected clones were spotted onto glass slides (GenePlorer™ Twinchip™, Digital Genomics). Sequences of spotted genes were annotated with Gene Ontology (GO) terms using Blast2GO (Conesa et al., 2005).

After the exposure test, the three fish in each treatment were dissected and their kidneys were collected. The kidney samples were immediately frozen in liquid nitrogen and were stored at −80 °C until use. Total RNA was extracted from the kidneys with TRIzol reagent (Invitrogen, Carlsbad, CA). RNA samples from control fish were pooled to use as a common reference. Second strand cDNA was synthesized and antisense RNA (aRNA) was amplified using a MessageAmp™ II aRNA Amplification Kit (Ambion, Austin, TX). Purified aRNA was reverse-transcribed with amino allyl-dUTP using an Amino Allyl cDNA Labeling Kit (Ambion), in which amino allyl cDNA was coupled with Cy3 (control or heavy oil-exposed) or Cy5 (common reference) monofunctional reactive dye (GE Healthcare UK, England, UK) and then mixed together. Excess Cy dye was removed using a QIAquick PCR purification kit (QIAGEN K. K., Tokyo, Japan). For hybridization, the following target solution was used: Cy dye-labeled DNA solution in 5× SSC, 0.1% SDS, 0.5 mg/ml PolyA (Invitrogen), 0.1 mg/ml salmon sperm DNA (Sigma–Aldrich Japan K.K., Tokyo, Japan), and 25% formamide. The target solution was heated at 95 °C for 2 min, and then placed on a glass slide. Three slides were used for each sample, and the slides were incubated at 60 °C for 17 h. Following hybridization, the slides were washed twice with 0.1% SDS in 2× SSC at 58 °C, and four times with 0.1% SDS in 0.1× SSC at room temperature, rinsed three times with 0.1% SSC, and then dried by centrifugation at 800 rpm for 3 min. The washed slides were then scanned using a fluor-image analyzer (FLA-8000, Fuji Photofilm Co. Ltd., Tokyo, Japan) at 532 nm (Cy3) and at 635 nm (Cy5).

2.3. Data analyses

Fluorescent intensities were quantified by ArrayGauge 2.1 (Fuji Photofilm). The intensities of the surrounding areas of each spot were used as background. Expression levels of each gene were represented as Cy3 (control or heavy oil-exposed): Cy5 (common reference) ratios, following normalization by the Locfit (LOWESS) function using TIGR MIDAS (version 2.19; Saeed et al., 2003). The expression data of each spot were validated by checking coefficients of variation (CVs) among three slides, which should be lower than 20%, and only data with acceptable variation were used for subsequent analyses.

Differences in gene expression levels between heavy oil-exposed and control groups were analyzed by the Student's *t*-test. Prior to analysis, gene expression levels were logarithmically transformed. All statistical analyses were performed using SPSS 15.0J (SPSS Japan, Tokyo, Japan). Differences were considered significant at $p < 0.05$.

Directed acyclic graphs were drawn to visualize the GO terms of a set of significantly up- or down-regulated genes using Blast2GO (Conesa et al., 2005).

3. Results and discussion

3.1. Microarray data

Gene expression analyses using our flounder cDNA array detected 84 (38 up-regulated and 46 down-regulated) genes affected by heavy oil exposure in the kidney of Japanese flounder. Prior to the statistical analyses, we calculated CVs for each spot among three independent slides (Table 1), and cut-off the data that varied widely (CV $\geq$ 20%) to remove unreliable data. Inter-slide CVs of the data were quite small, and only a few spots were removed from the subsequent statistical analyses. Therefore, the quantitative gene expression data generated by our flounder platform are reliable and highly reproducible.

Sets of the 38 up-regulated and 46 down-regulated genes were used to draw directed acyclic graphs showing "biological process" GO terms. Heavy oil exposure suppressed genes related to the immune response, iron ion homeostasis, proteolysis, and the cell cycle, among others (Fig. 1). On the other hand, the genes annotated as response to chemical stimulus, response to biotic stimulus, apoptosis, etc., were induced by the heavy oil treatment (Fig. 2). Details of the affected genes are discussed in the following sections.

3.2. Alteration of immune system-related genes

Among the 84 affected genes, we screened the genes whose expression levels were altered more than 1.5-fold (Tables 2 and 3). Several immune system-related genes, including immunoglobulin light chain, CD45, major histocompatibility complex (MHC) class II antigens, and macrophage colony-stimulating factor precursor, were down-regulated (Fig. 1). These results suggest that heavy oil exposure may suppress the immune function of flounders, especially the antibody generation and antigen presentation processes. Several studies have reported effects on the fish immune system by PAHs, although the effects are contradictory (reviewed by Reynaud and Deschaux, 2006) and depend on exposure route, types and concentrations of exposed PAHs, and fish species. However, our group reported that the bacterial counts increased in the skin mucus of heavy oil-exposed flounder (Song et al., 2008). This effect might be direct evidence for suppression of the immune system by heavy oil.

The members of a matrix metalloproteinase family, collagenase, metalloproteinase 9 and gelatinase, were also suppressed by the exposure to heavy oil (Fig. 3). The products of these genes play an important role in the degradation and remodeling of extracellular matrices, and are thought to be involved in inflammatory processes (Nagase, 1997; Noya et al., 1999). Suppression of these enzymes may cause pathogen resistance to decrease or be lost. Additionally, granulins, which are highly conserved growth factors, were also down-regulated by heavy oil exposure (Table 2). A granulin acts as a proliferator of goldfish (*Carassius auratus* L.) macrophages (Hanington et al., 2006). Therefore, decline of granulin function might lead to a decline of macrophage proliferation and differentiation.

On the other hand, the expression of beta-2 microglobulin, lysozyme and interleukin-8 (IL-8) were increased in the kidney of heavy oil-exposed flounders (Fig. 1). This pattern suggests that heavy oil exposure may not suppress overall immune function in flounder. Up-regulation of IL-8 might be a defensive response against bacterial infection, since bacterial counts increased in skin mucus of the same fish (Song et al., 2008). Chen et al. (2005) reported that bacterial infection induced IL-8 mRNA in the spleen of channel catfish (*Ictalurus punctatus*) and blue catfish (*I. furcatus*). It is well known that IL-8 can stimulate chemotaxis of neutrophils, which have a phagocytotic function. Since neutrophils produce lysozyme, increased IL-8 may attract neutrophils as a defensive response to infection, followed by up-regulation of lysozyme. From another viewpoint, since overactivated neutrophils release superoxide and consequently cause cellular damage (Knaapen et al., 2002), excessively induced IL-8 might cause cell damage in the kidneys of flounders exposed to heavy oil.

Gene expression analyses identified up- or down-regulation of immune system-related genes in the kidney of heavy oil-exposed flounders. In addition, our group observed increased bacterial counts in skin mucus and increased leukocyte counts in the blood of the same flounders used in the present study (Song et al., 2008). From these data, we hypothesize that heavy oil may suppress the pathogen resistance of exposed fish, leading to bacterial infection, and that the immune system may react against the infection, but it is unclear whether this response is ultimately effective.

Table 1
The number of spots defined by coefficient of variation (CV) and percentage of cut-off data

CV (%) range	Control			Heavy oil-exposed		
	Ind. 1	Ind. 2	Ind.3	Ind. 1	Ind. 2	Ind.3
<5	151	210	288	183	63	191
5–10	138	91	21	93	70	98
10–15	14	5	0	24	106	13
15–20	6	2	0	8	58	2
20≤	0	1	0	1	12	5
Removed spot (%)	0	0.3	0	0.3	3.9	1.6

3.3. Other affected genes

The expression of hemoglobin (Hb) beta chain was significantly suppressed by heavy oil exposure (Table 2; $p = 0.032$). Hb alpha chain expression in heavy oil-exposed fish was also 2.19-fold lower than that in control fish, although the difference was not statistically significant ($p = 0.057$). We reported that red blood cell counts were decreased in heavy oil-exposed flounder, compared to

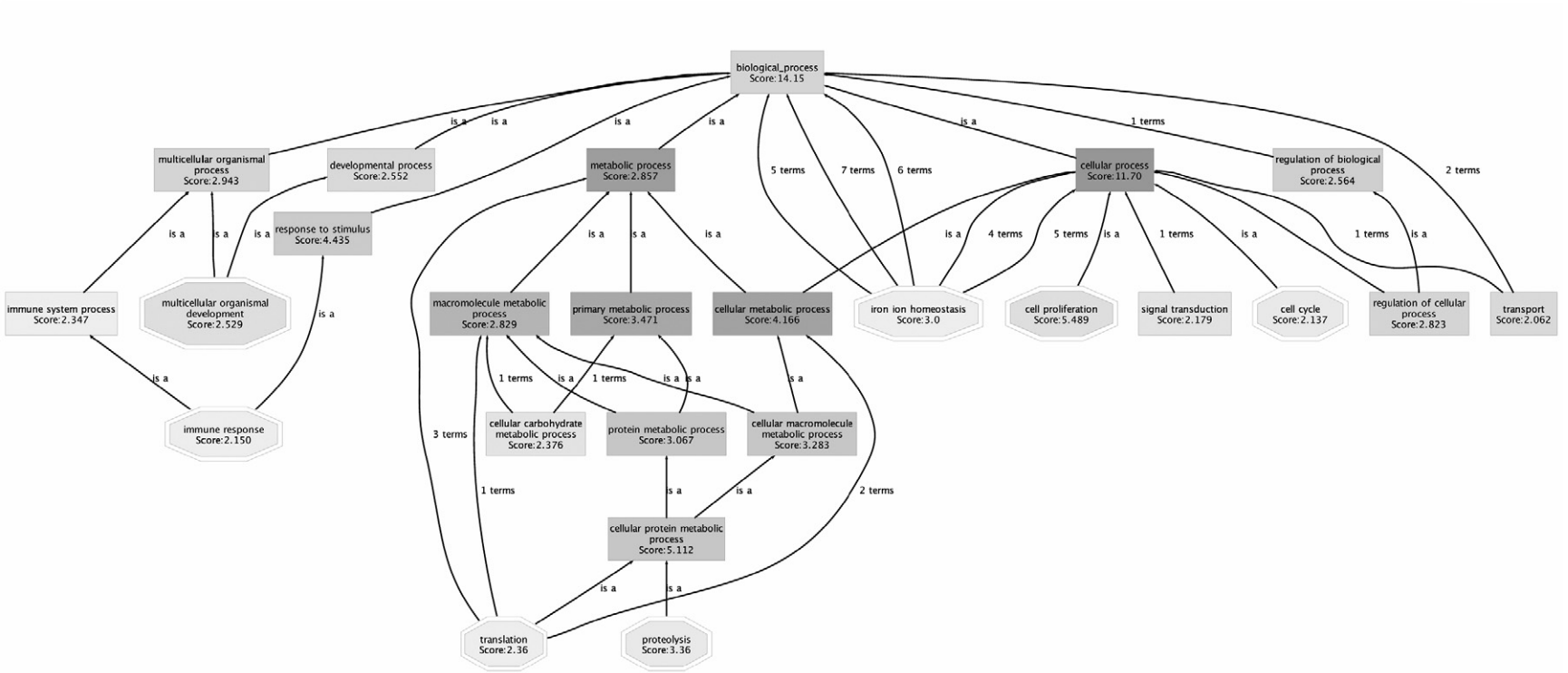

Fig. 1. Blast2GO directed acyclic graph showing "biological process" gene ontology terms of down-regulated genes ($p < 0.05$) in the kidneys of Japanese flounders exposed to heavy oil (Filters: number of sequences > 2, score > 2.0).

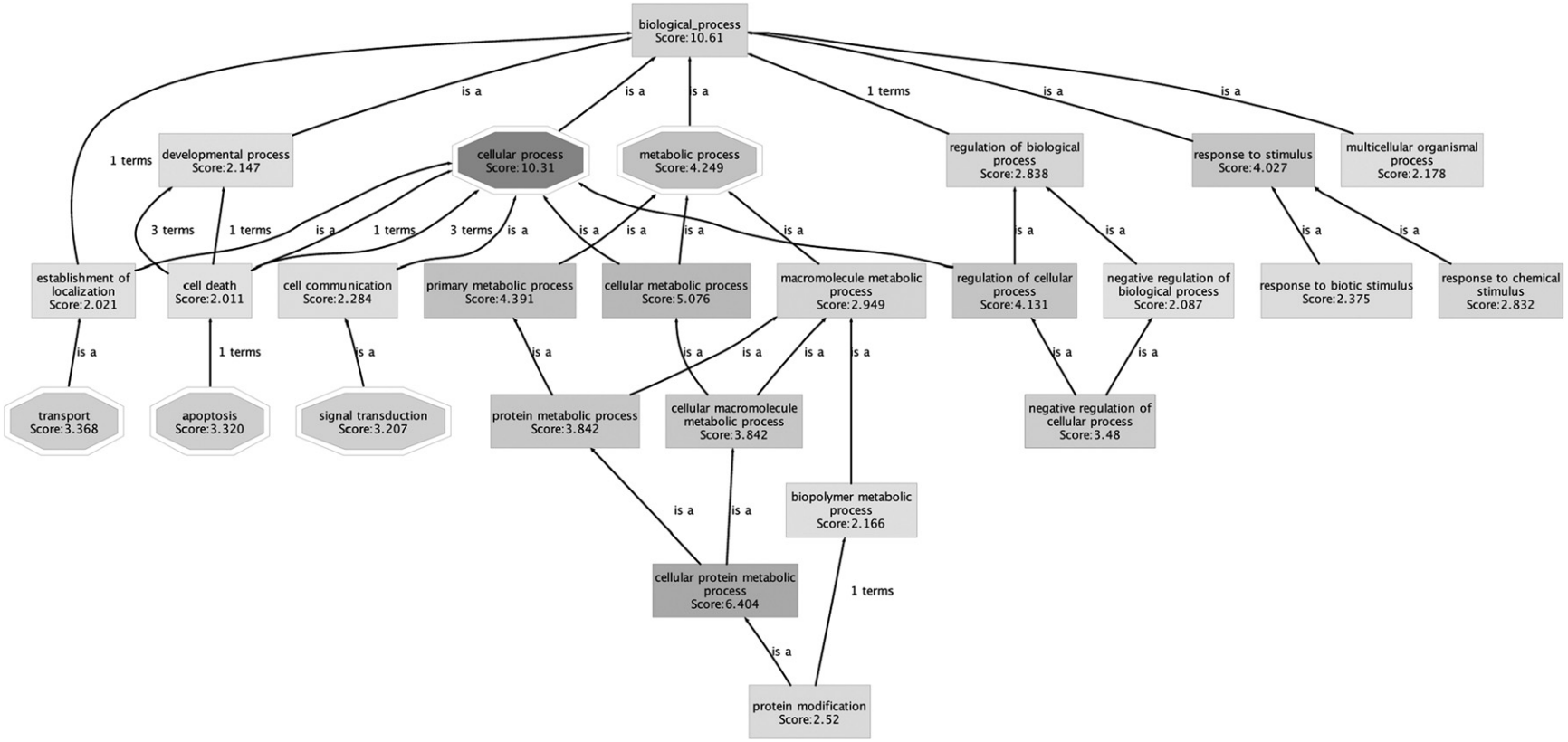

Fig. 2. Blast2GO directed acyclic graph showing "biological process" gene ontology terms of up-regulated genes ($p < 0.05$) in the kidneys of Japanese flounders exposed to heavy oil (Filters: number of sequences > 4, score > 2.0).

Table 2
Down-regulated genes (>1.5-fold) in the kidney of Japanese flounder exposed to heavy oil

Similar to	Control	Oil-exposed	Fold change
Serine protease I-1 [*Paralichthys olivaceus*]	1.11 ± 0.33	0.16 ± 0.04	−7.00
Nephrosin [*Oryzias latipes*]	1.06 ± 0.50	0.22 ± 0.05	−4.80
Retinoblastoma [*Limanda limanda*]	1.03 ± 0.32	0.22 ± 0.02	−4.58
Sialic-acid binding protein-4 [*Danio rerio*]	1.01 ± 0.35	0.28 ± 0.05	−3.62
Progranulin [*Oreochromis mossambicus*]	0.98 ± 0.31	0.28 ± 0.07	−3.54
Hemoglobin beta-A chain [*Siniperca chuatsi*]	0.89 ± 0.43	0.29 ± 0.04	−3.04
Collagenase [*Oryzias latipes*]	0.97 ± 0.33	0.33 ± 0.10	−2.91
Unnamed protein product [*Tetraodon nigroviridis*]	0.97 ± 0.29	0.35 ± 0.07	−2.80
Immunoglobulin light chain [*Takifugu rubripes*]	0.91 ± 0.40	0.36 ± 0.09	−2.57
Hypothetical protein LOC550588 [*Danio rerio*]	1.10 ± 0.12	0.46 ± 0.04	−2.41
Hypothetical protein LOC406266 [*Danio rerio*]	1.13 ± 0.43	0.49 ± 0.04	−2.30
Granulin-A precursor [*Danio rerio*]	1.16 ± 0.03	0.57 ± 0.11	−2.04
CD45 [*Takifugu rubripes*]	0.93 ± 0.24	0.46 ± 0.03	−2.02
Heparanase [*Danio rerio*]	0.99 ± 0.04	0.51 ± 0.13	−1.95
Unnamed protein product [*Tetraodon nigroviridis*]	0.94 ± 0.25	0.50 ± 0.07	−1.87
MHC class II A antigen [*Paralichthys olivaceus*]	1.06 ± 0.25	0.58 ± 0.12	−1.82
Matrix metalloproteinase 9 [*Paralichthys olivaceus*]	1.01 ± 0.23	0.56 ± 0.03	−1.81
Gelatinase [*Paralichthys olivaceus*]	1.01 ± 0.21	0.57 ± 0.06	−1.78
MHC class IIb antigen [*Paralichthys olivaceus*]	1.05 ± 0.14	0.60 ± 0.12	−1.76
Invariant chain-like protein 14-1 [*Oncorhynchus mykiss*]	0.98 ± 0.28	0.56 ± 0.02	−1.75
Macrophage colony-stimulating factor precursor [*Oncorhynchus mykiss*]	0.98 ± 0.08	0.57 ± 0.08	−1.73
Immunoglobulin light chain T2b constant region [*Takifugu rubripes*]	0.97 ± 0.22	0.60 ± 0.08	−1.61
Pim-1 protein kinase [*Mus musculus*]	0.97 ± 0.02	0.62 ± 0.17	−1.57

Table 3
Up-regulated genes (>1.5-fold) in the kidney of Japanese flounder exposed to heavy oil

Similar to	Control	Oil-exposed	Fold change
JAK1 tyrosine kinase [*Tetraodon fluviatilis*]	1.04 ± 0.07	1.58 ± 0.20	1.51
Beta-2 microglobulin [*Stizostedion vitreum*]	0.92 ± 0.12	1.39 ± 0.18	1.51
CCAAT/enhancer binding protein (C/EBP), delta [*Danio rerio*]	1.02 ± 0.09	1.58 ± 0.31	1.55
Syndecan binding protein [*Danio rerio*]	0.98 ± 0.09	1.53 ± 0.10	1.57
Epididymal secretory protein E1 [*Danio rerio*]	0.98 ± 0.07	1.56 ± 0.17	1.60
Cell division cycle protein 37 [*Tetraodon fluviatilis*]	1.06 ± 0.09	1.71 ± 0.36	1.62
hsc71 [*Paralichthys olivaceus*]	1.18 ± 0.17	1.92 ± 0.13	1.63
Cytochrome b [*Paralichthys olivaceus*]	0.85 ± 0.13	1.41 ± 0.25	1.65
Solute carrier family 25 alpha, member 5 [*Paralichthys olivaceus*]	1.17 ± 0.26	1.98 ± 0.27	1.69
Mvp protein [*Danio rerio*]	1.13 ± 0.06	1.96 ± 0.46	1.74
Unnamed protein product [*Tetraodon nigroviridis*]	1.09 ± 0.18	1.92 ± 0.17	1.76
K18, simple type I keratin [*Oncorhynchus mykiss*]	1.06 ± 0.12	1.93 ± 0.58	1.83
Proteasome subunit C10-11 [*Oncorhynchus mykiss*]	0.92 ± 0.16	1.74 ± 0.31	1.88
N-myc downstream regulated gene 1 protein, ndrg1 [*Danio rerio*]	1.12 ± 0.32	2.20 ± 0.48	1.97
Nucleoside phosphorylase [*Danio rerio*]	1.20 ± 0.17	2.36 ± 0.62	1.97
Beta cytoplasmic actin [*Pagrus major*]	1.04 ± 0.17	2.13 ± 0.30	2.06
Myeloperoxidase [*Siniperca chuatsi*]	1.12 ± 0.34	2.37 ± 0.32	2.12
Glutamine synthetase [*Oreochromis niloticus*]	1.04 ± 0.07	2.28 ± 0.19	2.19
Unnamed protein product [*Tetraodon nigroviridis*]	1.06 ± 0.06	2.36 ± 0.50	2.23
Chicken-type lysozyme [*Paralichthys olivaceus*]	1.12 ± 0.09	2.58 ± 0.44	2.30
Adipophilin (adipose differentiation-related protein) (ADRP) [*Danio rerio*]	1.03 ± 0.20	2.67 ± 0.19	2.60
Solute carrier family 6 (neurotransmitter transporter, GABA), member 13 [*Monodelphis domestica*]	1.02 ± 0.60	4.64 ± 2.20	4.56
Interleukin-8 [*Paralichthys olivaceus*]	1.02 ± 0.34	15.99 ± 9.02	15.63

control (Song et al., 2008). Previous studies on gene expression profiling in rainbow trout (*Oncorhynchus mykiss*) have demonstrated down-regulation of both Hb alpha and beta chain mRNAs in the kidney after exposure to carbon tetrachloride or pyrene (Krasnov et al., 2005), and of Hb beta in the liver after BaP exposure (Hook et al., 2006). Moreover, a decline in hemoglobin was found in winter flounder (*Pleuronectes americanus*) collected from PAH- and PCB-contaminated areas compared with those from a cleaner reference site (Khan, 2003). Thus, heavy oil exposure may suppress hemoglobin mRNA expression and subsequently result in declines in red blood cell counts.

Heat shock cognate 71 kDa protein (hsc71) and N-myc downstream regulated gene 1 protein (ndrg1) mRNA expression was induced in flounder kidney (Table 3). Up-regulation of these genes may be a stress response against

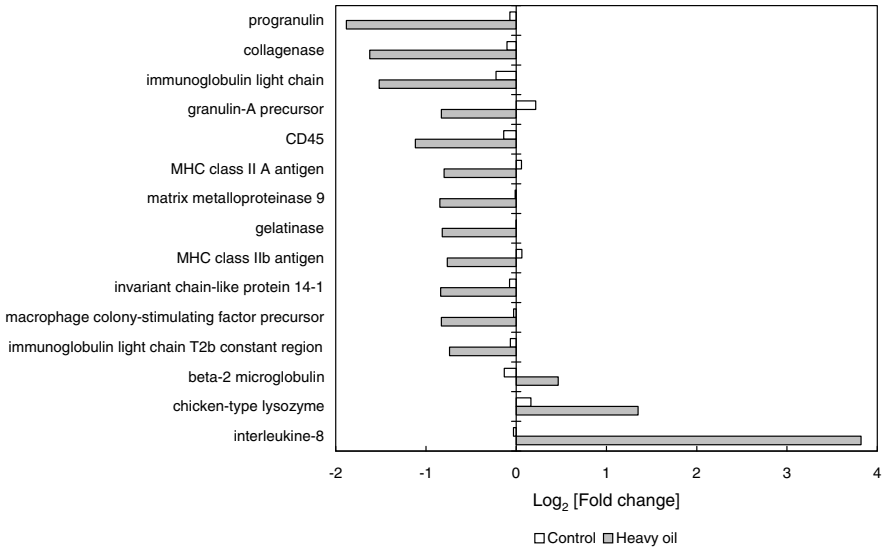

Fig. 3. Identification and fold change of immune system-related genes whose expression was significantly ($p < 0.05$) altered by heavy oil exposure.

heavy oil exposure. A previous study has demonstrated that exposures to carbon tetrachloride or pyrene cause induction of hsc71 in the liver and reduction of ndrg1 in the kidney of rainbow trout (Krasnov et al., 2005). In human cell lines, ndrg1 was induced by nickel exposure or under hypoxic conditions, but not by oxidative stress (Zhou et al., 1998; Salnikow et al., 2000). Even though it is still unclear how and by which chemical in heavy oil these genes were induced, both hsc71 and ndrg1 can be candidates for marker genes against heavy oil exposure in flounder.

The present study demonstrated transcriptome responses to heavy oil exposure in the kidney of Japanese flounder using our custom-made cDNA microarray. Immune system-related genes were mostly down-regulated by heavy oil, while IL-8 and lysozyme, which are involved in proinflammatory processes, were up-regulated. Considering the data that we have reported here and elsewhere (Song et al., 2008), pathogen resistance may be weakened in heavy oil-exposed fish, causing a subsequent bacterial infection, and then proinflammatory genes may be induced as a defensive response against the infection. Major tanker accidents spilled tens of thousands tons of oil (Stagg et al., 1998; Hayakawa et al., 2006; Davoodi and Claireaux, 2007), which may cause extremely high concentrations of oil contamination around the accident area. Even if dispersed oil were removed from the area soon after an accident, the effects of heavy oil on fish, especially indirect effects such as infectious disease, may occur.

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