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Screening of Ecotoxicant Responsive Genes and Expression Analysis of Benzo[a]pyrene-exposed Rockfish (*Sebastes schlgeli*)

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Abstract

Benzo[a]pyrene is a representative ecotoxicant in marine environment and a model compound of polycyclic aromatic hydrocarbons, which has an ability to bioaccumulate in aquatic organisms. This study aimed to identify molecular biomarkers suitable for assessing environmental pollution using a microarray technique. We examined the effects of benzo [a]pyrene on gene expressions in the rockfish, *Sebastes schlegeli*. We constructed the subtractive cDNA library with hepatic RNA from benzo[a]pyrene-exposed and non-exposed control fish. From the library 10,000 candidate clones were selected randomly and cDNA microarray was constructed. We determined benzo[a]pyrene-responsive genes using a high-density microarray. Statistical analysis showed that approximately 400 genes are significantly induced or reduced by benzo[a]pyrene treatment (2 μM). Especially gene expression changes of 4 candidate clones among the up- or down-regulated genes were investigated in 6, 12 and 24 hr BaP-exposed fish groups. Many methods have been developed to monitor marine environmental status, which depend on quantifying the levels of the toxic components in polluted seawater or on ecological accessing, such as species diversity or richness. However, those methods could not provide information on physiological or genetic changes induced by such environmental stresses. Comparing with the conventional methods, these data will propose that benzo[a] pyrene-responsive genes can be useful for biological risk assessment of polycyclic aromatic hydrocarbons on marine organism at molecular level.

Keywords: Benzo[a]pyrene, Biomarker, Differential gene expression, Subtractive cDNA library

Anthropogenic contamination such as sewage containing persistent organic pollutants and a variety of toxic chemicals from land runoff is the representative environmental stress in marine ecosystem. In coastal area, extrinsic endocrine disrupting materials such as polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) were widely distributed. Benzo[a]pyrene (BaP) among PAHs, is a representative ecotoxicant and has been reported bioaccumulative potential in many organisms resulting in DNA damage, endocrine disruption, and reproductive disturbance[1]. DNA damage induced at early life stage of an organism inhibits the development to adult, which might cause disturbance of ecosystem along the food chain as well as serious economic loss in fisheries. In addition, the extinction of a species in marine ecosystem may affect its community. Therefore, it is considerably necessary to detect the biological effects of pollutant on marine organisms under low level of contamination as well as the health status of marine ecosystem.

Rockfish, *Sebastes schlegeli*, distribute in shallow coastal area of Korea and Japan. This species is one of the aquacultural fishes and is important both in market and table in Korea. In this study we choose the rockfish because of its economic value as food resource and considerable easiness on handling and obtainment as well.

Microarray technology is a strong tool that can be applied to obtain toxicological insight of organisms massively and to identify biomarkers for exterior stress responsiveness. In addition, their gene expression changes can make a potential prediction for physiological aspect or sequential events caused by specific stressors. In this study, we aimed to explore the ecotoxicant-responsive genes in rockfish, *Sebastes schlegeli*, using microarray technique and to obtain the molecular-level information investigating the changes of gene expressions in fish affected by exposure to bioaccumulative ecotoxicants.

Screening of Differentially Expressed Genes

This study aimed to discover the genes which transcriptions were changed by the environmental toxicant BaP and to develop a simple and efficient system such as cDNA chip that could be used to assess the contamination at PAH-impacted sites. As its first

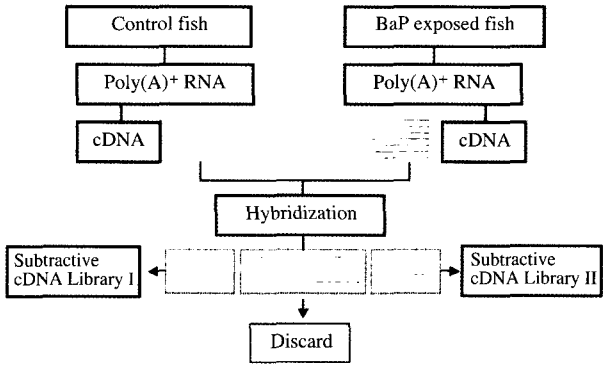

Fig. 1. Construction of subtractive cDNA library. Poly (A)$^+$ RNA was extracted from liver of non-exposed control and BaP exposed fish. After reverse transcription to cDNA, they were hybridized for subtraction according to the manufacturer's instruction. Subtractive cDNA library I is a library of down-regulated genes and II is a library of up-regulated genes.

step, we constructed two subtractive cDNA libraries as shown in Figure 1. Subtractive library I is down-regulated genes from *Sebastes schlegeli* exposed to BaP and subtractive library II is up-regulated genes by BaP exposure. To confirm a successful library construction, 2,000 clones of each library were analyzed by random sequencing then we investigated the repetition of clone annotation. Each 5,000 clones from subtractive library I and II were used to construction of cDNA chip. Each cDNA chip was hybridized with 0 hr or 24 hr BaP-exposed fish RNA (Fig. 2) and the data from hybridization with 6 and 12 hr BaP-exposure fish RNA were compared and analyzed (Fig. 3). As a result, we obtained 31 clones whose expressions significantly increased by BaP exposure comparing with those of unexposed control and 11 clones whose expressions significantly decreased by BaP exposure comparing with those of unexposed control (Table 1, 2).

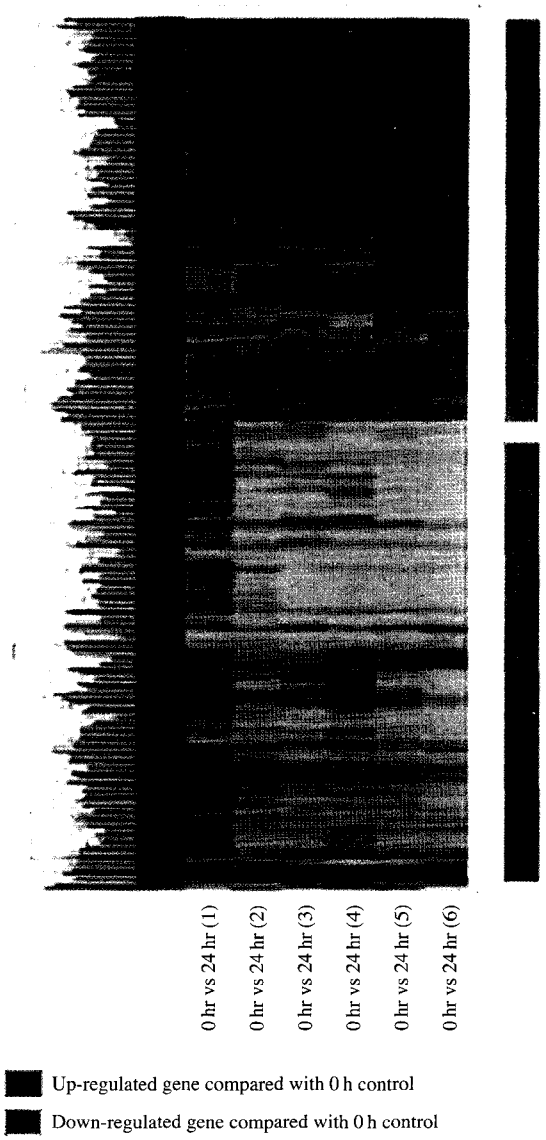

Fig. 2. Hierarchical cluster image showing the differentially expressed genes in liver from BaP-exposed rockfish.

Gene Expression Changes by BaP Exposure

Among the up- or down- regulated gene clones, four clones were analyzed to investigate their expression changes by BaP exposure duration (6, 12 and 24 hr) (Fig. 4). The result displayed significant change of gene expression in accordance with BaP-exposure time. The patterns of induction or repression showed proportional relation to BaP exposure time and the differential gene expressions in those 4 fragments were observed with the expression level change between approximately 2 and 3-fold at 24 hr post-BaP exposure, compared with 0 hr control group.

Clone 1, which nucleotide sequence showed homology to betaine homocysteine methyltransferase in *Mus musculus* (GenBank accession No. BC013515) was repressed gradually and approximately 2-fold down regulated at 24 hr post-exposure compared with 0 hr control. According to the recent report, betaine homocysteine methyltransferase was identified as a down-regulated gene in rat liver cirrhosis and most likely involved in dysregulation of homocystein level[2,3]. It has been known that homocysteine levels are elevated in liver cirrhosis and hepatic encephalopathy incorporates neuropsychiatric abnormalities with liver dysfunction. Also it has been focused on the molecular mechanisms involved in the pathogenesis

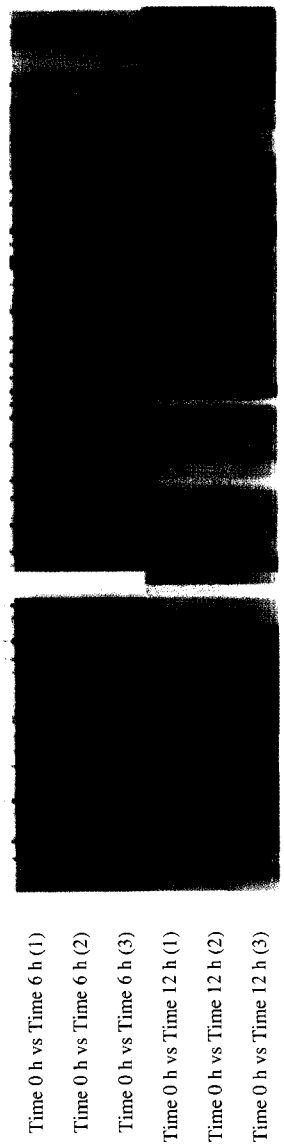

Fig. 3. Hierarchical clustering of gene expression in liver from rockfish exposed to BaP for 6 and 12 hr.

Table 1. Up-regulated genes in *Sebastes schlegeli* exposed to BaP for 24 hr.

Genbank No.	Gene name
AF026525	Antifreeze protein precursor
NM-009911	Chemokine (C-X-C motif) receptor 4 (Cxcr4)
AF151726	Putative MtN3-like protein
BA000027	MHC Class I Region
AY327035	Cytochrome oxidase subunit I mRNA
AJ749809	Piscicida trpA gene for putative transposase, clone pRDA33
AJ749800	Piscicida partial coi genes for putative cytochrome C oxidase proteins
S72345	cAMP-dependent protein kinase RI beta subunit (exons I and II, promoter)
BX088694	A novel protein similar to vertebrate discoidin domain receptor family, member 2, a novel gene and two CpG islands
AF056314	Gonadotropin-releasing hormone gene
AJ293850	Putative positive transcription regulator EVGA
AJ534345	Putative glyoxylate pathway regulator (gpr)
AY686211	Gelsolin gene
AB037867	Fucolectin-1
AJ290944	A stretch regulated skeletal muscle protein
AJ749800	Piscicida partial coi genes for putative cytochrome C oxidase proteins
AB047592	Alpha-crystallin-related protein
AY713945	MHC class II antigen gene cluster
AF212926	Allatotropin neuropeptide precursor
AF212039	Alpha-interferon inducible protein
U93881	Penicillin G acylase precursor
AY521670	Ceruloplasmin
AB111382	Fructose-1, 6-bisphosphate aldolase
AB075199	Warm-temperature-acclimation-related-65 kDa -protein-like-protein
NM080888	BCL2/adenovirus E1B 19 kDa-interacting protein
AY575953	Glycine max beta-carotene hydroxylase
AY541450	Translation initiation factor eIF-2B beta subunit gene
AF300706	Serum amyloid A protein
DLU78316	Cytochrome P450 1A
AJ973596	polyglutamine binding protein variant 4 (PQBP1 gene)
DQ387060	NAD(P)H dehydrogenase quinone 1 mRNA

of hepatic encephalopathy with liver dysfunction incorporating with betaine homocysteine methyltransferase and therapy of hepatic encephalopathy to reduce ammonia generation and increase its detoxification[3].

Clone 2 was identified homologous to *Oryzias latipes* warm-temperature-acclimation-related 65 kDa protein-like-protein (WAP) (GenBank accession No. AB075199). Its expression was increased at 6 hr post -BaP exposure with approximately 1.5-fold expression change and showed approximately 2-fold up-regulation at 24 hr post-exposure. In similar study using DD-PCR method to identify hepatic genes responsive to thermal stress in *Fundulus heteroclitus*, genes that differed in expression between fish populations from different thermal environments were discovered and they showed high homology to genes of known function such as glucokinase, serine-threonine kinase 10, glycogen synthase kinase, and warm acclimation-related protein[4]. Those genes significantly changed in expression between populations from different thermal environments and especially expression of WAP was approximately 8-fold higher in Southern than in Northern fish, consistent with a previously suggested role for this gene in thermal accli-

Table 2. Down-regulated genes in *Sebastes schlegeli* exposed to BaP for 24 hr.

Genbank No.	Gene name
BC013515	Betaine-homocysteine methyltransferase
CAC29154	Complement component C3
AAQ63949	Putative transferrin
XP_42367	Epidermal lipoxygenase; lipoxygenase-3
AAC37673	Arachidonate 5-lipoxygenase
AAD39096	15S-lipoxygenase type 2
BAA88899	Complement component C7
AAM73701	C1q-like adipose specific protein
NP_998300	Cytochrome b5
NP_695213	Arachidonate 15-lipoxygenase, second type
CAA70062	12-lipoxygenase

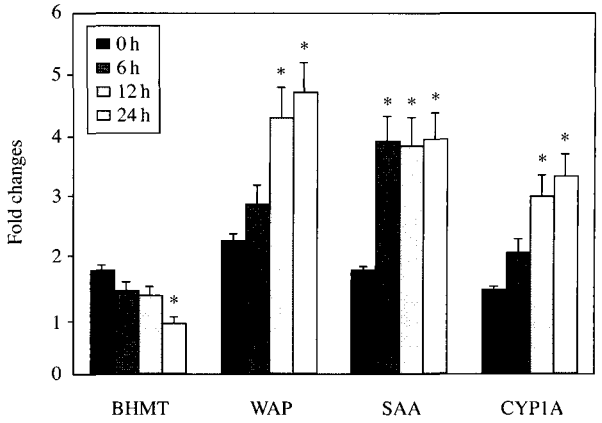

Fig. 4. Quantitative comparison of gene expression levels in different duration of BaP exposure. BHMT, betaine-homocysteine methyltransferase; WAP, warm-temperature-acclimation-related 65 kDa protein like protein; SAA, serum amyloid A; CYP1A; cytochrome P450 1A. *Significantly different from 0 hr ($p < 0.05$).

mation or adaptation to extrinsic stresses in fish[4]. In this study, a fragment showing homology to WAP exhibited similar expression pattern by ecotoxicant exposure without thermal change. This result could suggest that exposure to ecotoxicant might cause various damages in the exposed organisms such as incomplete transcription, cellular dysfunction, or metabolic imbalance like as the moment an organism was exposed to chilling or heating stress.

Clone 3 showing 2.5-fold higher expression in 24 hr BaP exposure group, had homology to *Holothuria glaberrima* serum amyloid A protein (GenBank accession No. AF300706). Comparing with 0 hr control, its expression in all BaP-exposed fish groups was drastically increased. Serum amyloid A (SAA) is known as a superfamily of acute-phase proteins. The level of SAA in blood increases dramatically in response to tissue injury and inflammation, influencing cell adhesion, migration, proliferation and aggregation[5]. In human, SAA was reported as the precursor to the amyloid A protein found in deposits of reactive amyloid and the amyloid deposition might contribute to the pathogenesis such as Alzheimer's disease[6]. In the independent studies on two heterochonch bivalves (*Mya arenaria*, *Mactromeris polynyma*) and oyster (*Crassostrea virginica*) exposed to PAHs and high level of organochlorine compound, respectively, phagocytosis by hemocytes in both heterochonch bivalves was significantly suppressed[7]. And the results deduced from *C. virginica* exposed to organochlorine compound suggested that aquatic contaminant could interact rapidly with hemocytes to produce a partially reversible immunotoxicological lesion[8].

The last clone 4, which level of expression was proportionally increased after BaP exposure, showed high similarity to *Dicentrarchus labrax* cytochrome P450 1A (GenBank accession No. DLU78316). CYP1 gene family is efficient metabolizers of PAHs and was induced in fish by various PAHs[9]. This enzyme is involved in an NADPH-dependent electron transport pathway. It oxidizes a variety of structurally unrelated compounds, including steroids, fatty acids, and xenobiotics[10]. CYP1A protein was found to be significantly higher at contaminated sites in response to PAHs and PCBs in the mussel *Mytilus* sp.[11], in the sole *Pleuronectes vetulus*[12], and in the salmon *Salmo salar*[13].

Discussion

In conclusion, we constructed subtractive cDNA library and successfully isolated the differentially expressed in response to the existence of BaP through the microarray analyses. We investigated the expression changes of those candidate genes in various exposure durations. Particularly, four genes were presumed to be the *Sebastes* homologues of betaine homocysteine methyltransferase, warm-temperature-acclimation-related protein, serum amyloid A protein, and cytochrome P450 1A, respectively. All those genes are well known to relate to pathogenesis, extrinsic stresses, and catalytic metabolites. Considering the expected function of the isolated genes, the subtractive cDNA library construction and microarray data might be strong and effective in isolation of the differentially expressed genes and provided the knowledge regarding gene expressions by toxicant impact. Furthermore, this result might be useful for the development of biomarkers to assess marine envi-

ronmental stress or contamination.

Methods

Animal

Rockfish (300-350 g) were obtained from Sunghae Fisheries Cooperative (Geoje, Korea) and acclimated for 2 weeks in an aquatic facility. Three groups (n=3, each group) were assigned to filtered seawater containing 2 μM of BaP [dissolved in DMSO] (Sigma) for 6, 12 and 24 hr, respectively. A control group was maintained in filtered seawater. RNAs were extracted from fish livers using Trizol reagent (Sigma) following the manufacturer's instruction. RNAs from the livers of the fish assigned to seawater were used as control.

Subtractive cDNA Library Construction

We constructed subtractive cDNA library to identify the differentially expressed genes responding to the chemical contaminant such as BaP. RNA was extracted from liver of control fish and BaP-exposed fish and each subtractive cDNA libraries were constructed by using PCR-select cDNA subtraction kit (BD Biosciences) following the manufacturer's direction. Sequencing of positive clones was carried out with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

Preparation of cDNA Probe and Microarray Hybridization

The cDNA microarray containing 10,000 clones of fish cDNA proceeded according to the general procedures by GenomicTree Inc. The synthesis of target cDNA probes and hybridization were performed according to previously described[14]. Each 50 μg total RNA was reverse transcribed in the presence of Cy3 or Cy5-dUTP (NEN Life Sciences) at 42°C for 2 hr. Control RNA was labeled with fluorescent Cy3-dUTP and test RNA was labeled with fluorescent Cy5-dUTP. Both the Cy3 and Cy5-labeled cDNA were purified using PCR purification kit (Qiagen) as recommended by manufacturer. The purified cDNA was resuspended in 100 μL of hybridization solution and the hybridization mixtures were heated at 100°C for 2-3 min and directly pipetted onto microarrays. The arrays hybridized at 42°C for 12-16 hr in the humidified hybridization chamber (GenomicTree Inc., Korea). The hybridized miroarrays were washed with 2 × SSC/0.1% SDS for 5 min, 0.1 × SSC/0.1% SDS for 10 min, and 0.1 × SSC for 2 min two times. The washed microarrays were immediately dried using the microarray centrifuge (GenomicTree Inc., Korea).

Data Acquisition and Analysis

After hybridization, microarray slides were imaged using laser scanner (Axon 4000B, Axon Instruments Inc., Foster, CA, USA). The signal and background fluorescence intensities were calculated for each probe spot by averaging the intensities of every pixel inside the target region using GenePix Pro 4.0 software (Axon Instruments Inc., Foster, CA, USA). Spots were excluded from analysis due to obvious abnormalities. All data normalization, statistical analysis and cluster analysis were performed using GeneSpring 7.2 (Agilent, CA, USA). The fold change was calculated by dividing the median of normalized red channel intensity by the median of normalized green channel intensity. The ratios of fold changes across conditions were calculated by comparing of the values of fold changes between given conditions. The ANOVA test (parametric) was performed at the p values 0.05 to find genes that differentially expressed across conditions. Unsupervised hierarchical clustering was performed by similarity measurements based on Pearson correlations around zero.

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