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Molecular Parameters for Assessing Marine Biotoxicity: Gene Expressions of Rockfish (*Sebastes schlegeli*) Exposed to Polycyclic Aromatic Hydrocarbons

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Abstract

Environmental and anthropogenic changes affect the health and stability of marine ecosystem. In this study we aimed to identify molecular biomarkers for ecotoxicological pollutants risk assessment in the rockfish (*Sebastes schlegeli*). We designed primers based on conserved sequences by multiple alignments of target genes from related species, and cloned the partial cDNAs of cytochrome P450 (CYP1A1), glutathione S-transferase (GST), metallothionein (MT), superoxide dismutase (SOD), ubiquitin (UB), vitellogenin (VTG) and β-actin by reverse transcription polymerase chain reaction (RT-PCR) from *S. schlegeli*. Northern blot results indicated that these six genes expressions were significantly induced by benzo[a]pyrene (BaP, 1 μM) and that the level of each of their transcripts increased in BaP-exposed rockfish in a time-dependent manner. This study suggests that transcriptional changes in these six genes may be used for monitoring environmental exposure to polycyclic aromatic hydrocarbons (PAHs).

Keywords: *Sebastes schlegeli*, Environmental stress, Benzo[a]pyrene, Biomarkers, Gene expression

The marine environment is continuously being affected by a large number anthropogenic contaminants and stressors that threaten its stability. This is particularly true in densely populated and massively developed coastal areas such as the areas involved in shipbuilding or marine engineering, and the areas contaminated by various types of land runoffs or sewage. Currently, various methods of biotoxicity assessment have been developed, for example, the analyses of specific enzyme activity variations involved in cellular defense[1], the use of protein chip technology to follow proteomic profiles changes in organisms exposed to environmental toxicants[2], and the direct determination of DNA strand breaks or levels of PAH metabolites in the polluted marine organisms[3]. However, investigations on transcriptional changes in representative genes, which are known to respond to abiotic stressors, are rare in marine organisms. In this study we aimed to develop the integrated biomarkers through the analyses of transcriptional changes induced by ecotoxicant and as a first step for this approach, we isolated six genes which are known to be related with oxidative stress, heavy metal toxicity, reproductive toxicity, and genotoxicity from rockfish, *Sebastes schlegeli*. Rockfish, *S. schlegeli*, distribute in coastal area of Korea and Japan and we choose the rockfish as an experimental animal because of its importance as food resource and considerable easiness on handling and obtainment (Figure 1).

The integrated use of biomarkers of exposure and knowledge of the effects of oxidative stress, heavy metal toxicity, reproductive toxicity, and genotoxicity in living marine organisms may help us to better interpret the impact of pollutants on the marine coastal environment. The molecular tools introduced in this study can provide information on the health of species and thus contribute to our knowledge of marine ecosystem and provide information on the pollution status of coastal regions.

Cloning of CYP1A1, GST, MT, SOD, UB and VTG

To clone the target genes, nucleotide sequences were selected in conserved regions as primers after performing multiple alignments of each gene from the known sequences of related species [*Dicentrarchus labrax* (CYP1A1), *Pleuronectes platessa* (GST), *Sparus aurata* (MT), *Acanthopagrus schlegelii* (SOD), *Pagrus major* (UB), *Sillago japonica* (VTG)]. The primers used to amplify the targeted genes are listed

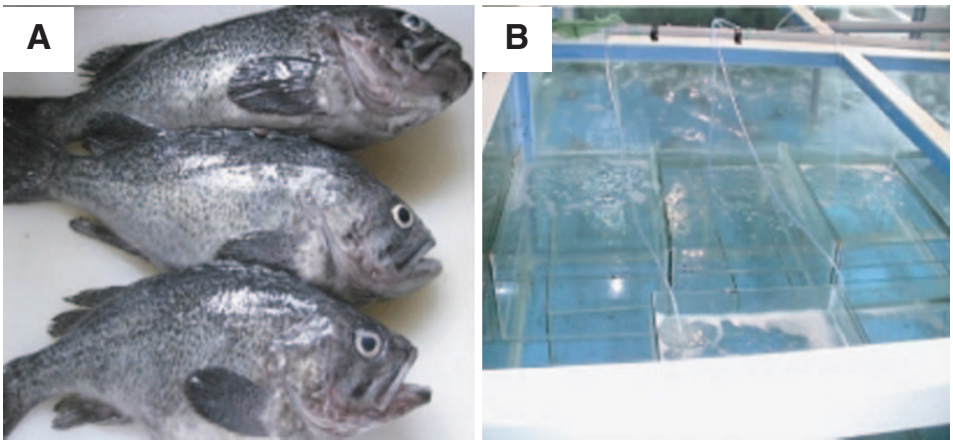

Figure 1. Aquacultured rockfish and experimental condition. Rockfish (*S. schlegeli*) used in this study were acclimated for 2 weeks in an aquatic facility after purchasing (A) and were exposed to chemical in glass water tanks with 24 hrs aeration (B).

Table 1. List of primers used for reverse transcription polymerase chain reactions.

Gene	Primer	Sequence	Reference
CYP1A	CYPUP CYPDN	5′-gaagctagatgagaacgcgaa-3′ 5′-gatgtgcaatgagggatagtga-3′	*Dicentrarchus labrax* (U78316)
GST	GSTUP-1 GSTDN-1 GSTUP-1NE GSTDN-1NE	5′-gaagaacctgcagggctaca-3′ 5′-gtcaggccctcaaacatgcg-3′ 5′-gaacctgcagggctacaacc-3′ 5′-ggccctcaaacatgcgttgg-3′	*Pleuronectes platessa* (X95200)
MT	MTUP-1 MTDN-1 MTUP-1NE MTDN-1NE	5′-gacccttgcgagtgctctaa-3′ 5′-gcagcagctagtgtcgcacg-3′ 5′-gcgagtgctctaagactgga-3′ 5′-gtgtcgcacgtcttcccttt-3′	*Sparus aurata* (U58774)
SOD	SODUP-1 SODDN-1	5′-gaagctcacaggagaaatca-3′ 5′-gccagacgtccaccagcgtt-3′	*Acanthopagrus schlegelii* (AJ000249)
UB	UBUP UBDN UBUP-NE UBDN-NE	5′-gcagatcttcgtgaaaac-3′ 5′-gcgcaggttgttggtgtg-3′ 5′-accatcaccctcgaggt-3′ 5′-ttcttcttgcgacagtt-3′	*Pagrus major* (AY190746)
VTG	VTGUP-2 VTGDN-2 VTGUP-2NE VTGDN-2NE	5′-gaggaacatcgcaaagaagg-3′ 5′-ggtgtccctcagctcttact-3′ 5′-ggagcccagaatgatccagg-3′ 5′-gctcttactccaacctccag-3′	*Sillago japonica* (AB081299)
β-ACT	ACTUP ACTDN	5′-gatctggcatcacaccttctacaa-3′ 5′-tacatggcaggggtgttgaaggtc-3′	*Sebastes schlegeli* (AY166590)

in Table 1.

Gene Expression Changes Induced by BaP Exposure

Using the primers listed in Table 1, we amplified PCR products of target genes in *S. schlegeli* and cloned partial cDNA encoding cytochrome P450 (1A1) (GenBank accession no. AY568556), glutathione S-transferase (AY771323), metallothionein (AY771322), superoxide dismutase (AY771324), ubiquitin (AY803760), and vitellogenin (AY771325) from rockfish as molecular markers with a view towards using them as markers for accessing marine environmental status, using β-actin (AY166590) as an internal control. We determined their nucleotide sequences and investigated their gene expression changes by northern blotting in rockfish exposed to BaP, a representative ecotoxicant to be bioaccumulated in aquatic organisms[4] for 0, 6, 12 and 24 hrs (Figure 2).

CYP1 gene family is efficient metabolizers of PAHs and induced in fish by different PAHs[5]. CYP1A -immunopositive protein was found to be significantly higher at contaminated sites in response to PAHs and PCBs in the mussel *Mytilus* sp.[6], in the sole *Pleu-*

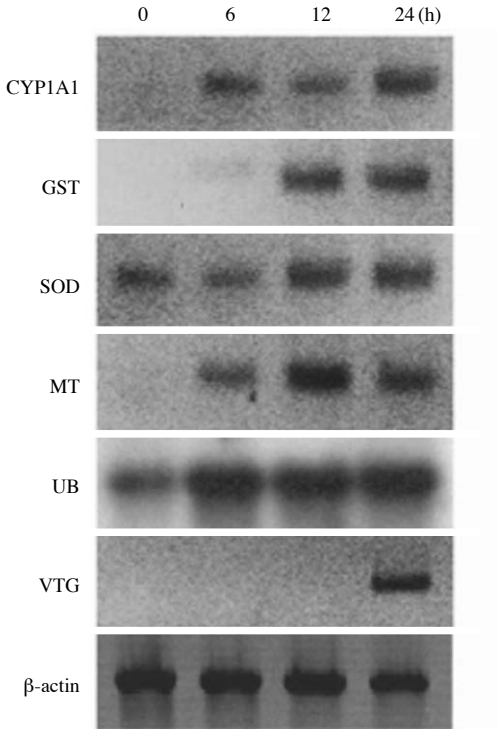

Figure 2. Gene expressions of CYP1A1, GST, MT, SOD, UB and VTG in BaP-exposed rockfish. RNAs were obtained from the livers of BaP-exposed and non-exposed rockfish. Total RNAs (20 μg) were separated by electrophoresis, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled cDNA of CYP1A1, GST, MT, SOD, UB and VTG. The times 0 to 24 hrs refer to the duration of BaP exposure. For internal control, β-actin cDNA probe hybridized with RNA gel blot was used. Each membrane was stripped and rehybridized with β-actin probe.

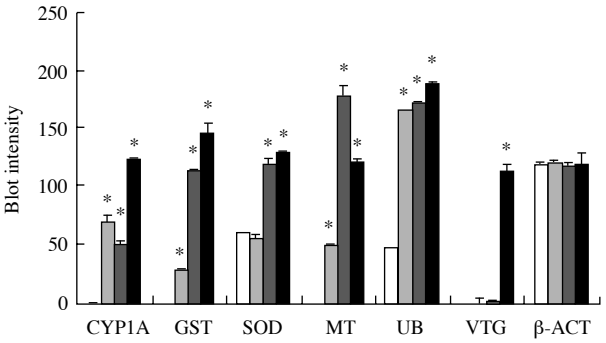

Figure 3. Quantitative analyses of expression changes of CYP1A1, GST, MT, SOD, UB and VTG in BaP-exposed rockfish. The northern blot images of CYP1A1, GST, MT, SOD, UB and VTG were analyzed using NIH Scion Image System and the gene expression changes were presented as the blot intensities. White, 0 hrs; line, 6 hrs; dot, 12 hrs; black, 24 hrs. *Significantly different from each 0 hrs group ($P < 0.05$).

ronectes vetulus[7] and in the salmon *Salmo salar*[8]. In this study, a 303 bp partial cDNA of CYP1A1 was cloned and shared 91% identity with cytochrome P450 1A (CYP1A) mRNA of *Dicentrarchus labrax* (GenBank accession no. U78316), 89% identity with that of *Micropterus salmoides* (AY619695), and 89% identity with that of *Chaetodon capistratus* (U19855). Northern blot showed that the CYP1A1 transcript was barely detectable in the 0 hrs control group but was significantly induced in BaP exposed groups. mRNA intensity of northern blot analyzed by Scion image program was presented as blot intensity. The level of CYP1A1 mRNA was elevated in all BaP-exposed groups compared with 0 hrs control group and it was shown the most in 24 hrs exposed group (Figure 3).

Glutathione S-transferase is a family of enzymes that utilize glutathione in reactions that contribute to the transformation of a wide range of compounds, including carcinogens, xenobiotics, and the products of oxidative stress. These enzymes play key roles in the detoxification of such substances[9]. In the current study, the nucleotide sequence of a 242 bp GST cDNA fragment from *S. schlegeli* showed 88% identity with *Pleuronectes platessa* glutathione S-transferase mRNA (GenBank accession no. X95200), 85% identity with that of *Platichthys flesus* (AJ3104280), and 81% identity with that of *Micropterus salmoides* (AY335905). The GST transcript level in *S. schlegeli* was upregulated in response to BaP stress comparing the control group, and significantly higher levels of GST mRNA were observed after 12 hrs and 24 hrs of exposure and barely detected in 0 and 6 hr exposed groups.

Metallothionein (MT) is a small stress response protein that can be induced by exposure to heavy metal cations, oxidative stressors, and acute phase cytokines that mediate inflammation[10]. In vitro evidence demonstrates that metallothioneins might act as antioxidants and that they can protect against metal toxicity[11]. Analysis of metallothioneins has revealed that certain MT isoforms preferentially associate with cadmium or with copper and zinc in the American oyster[12]. In the present study, a partial cDNA *S. schlegeli* MT of 174 bp in length was cloned and found to share 92% identity with *Sparus aurata* methallothionein mRNA (GenBank accession no. SAU58774), 92% identity with that of *Gobius niger* (AF520609), and 92% with that of *Chrysophrys major* (AB039668). The MT gene in *S. schlegeli* was significantly expressed in all BaP-exposed groups, but was not detected in the control group. This MT signal corresponded to a

similar upregulation of CYP1A1, suggesting its utility as an acute indicator of BaP-stress response in *S. schlegeli*.

Aerobic organisms are protected against oxidative stress by antioxidant systems which mobilize enzymes such as the Cu/Zn superoxide dismutase[13]. Superoxide dismutases catalyze the reaction between superoxide ions and two protons to form hydrogen peroxide and O_2[14], and accumulate in response to oxidative stress. Superoxide dismutases are one of the main antioxidant defense pathways and an index that the cell is responding to oxidative stress. A 330 bp cDNA fragment of SOD from *S. schlegeli* was found to share 93% identity with *Acanthopagrus aschlegeli* Cu/Zn-superoxide dismutase mRNA (GenBank accession no. AJ000249), 92% identity with that of *Oplegnathus fasciatus* (AY613390), and 92% identity with that of *Pagrus major* (AF329278). Northern blot data showed that SOD gene was expressed in all BaP-exposed groups in this study, whereas CYP1A1, GST, MT, and VTG genes were not detected in the control. The SOD transcript level was elevated significantly in 12 hrs and 24 hrs BaP-exposed groups comparing the non-exposed group ($P < 0.05$).

Ubiquitin is a small, 76-amino acid protein, which can be covalently attached to target proteins destined for removal. Ubiquitination serves as a recognition signal for degradation by proteasome[15]. Short-lived or unnecessary proteins are broken down into short peptides by this massive protein complex. The proteasome is, thus, responsible for numerous important biological processes, including cell cycle, differentiation, stress response, neuronal modulation, secretion, DNA repair, transcriptional regulation, long-term memory, circadian rhythms, immune response, and organelle biogenesis[16]. Ubiquitin was identified as a major player in the DNA damage repair system[17] and also implicated in plasma membrane endocytosis, followed by vacuole degradation, in yeast[18]. In this study, we cloned 308 bp of ubiquitin-encoding partial cDNA in *S. schlegeli*, and investigated the changes of gene expression by exposure to BaP. The nucleotide sequence was found to share 99% identity with *Oncorhynchus mykiss* ubiquitin mRNA (GenBank accession no. AB036060), and 95% identity with *Pagrus major* ubiquitin mRNA (AY190746). In northern blot result, ubiquitin mRNA was detected in all four groups including non-exposed control, showing the similar feature of SOD expression. The level of ubiquitin transcript was elevated significantly in 6 hrs, 12 hrs, and 24 hrs BaP-exposed groups compared with non-exposed control group ($P < 0.05$). Among the target genes in this study, UB gene showed the robust expression changes responding toxicant chemical.

The synthesis of egg yolk protein precursor VTG in the liver of female fish is strictly controlled by estrogens[19]. Once estrogen has bound to the estrogen receptors in the fish liver[20], the hormone-receptor complex binds with estrogen-response elements to induce VTG transcription[21]. In females, VTG is sequestered from the blood by growing oocytes. However, abnormally elevated amounts of VTG were observed in males exposed to endocrine-disrupting substances, which stimulate the ER linked to the induction of VTG[22,23]. In this study, the partial cDNA of *S. schlegeli* VTG was of 391 bp and the nucleotide sequence showed 89% identity with *Sillago japonica* vitellogenin mRNA (GenBank accession no. AB081299), 89% identity with that of *Rivulus marmoratus* (AY279214). VTG mRNA was only detected in livers of rockfish exposed to BaP for 24 hrs. These data indicate that potential reproductive toxicity might be induced by a class of compounds known as PAHs and that the elevated VTG expression signals the exposure of an organism to an environmental stressor.

Discussion

This study was initiated to identify molecular biomarkers suitable for assessing environmental pollution at different sites along the Masan coastline (South Sea, Korea). Specifically, we examined BaP exposure levels (data not shown) and the effects of BaP on the expressions of representative stress-related genes in the rockfish, *Sebastes schlegeli*. 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, these methods do not provide information on physiological or genetic changes induced by such environmental stresses. In this study BaP was found to alter the expressions of the seven genes which functions have been well known in eukaryotic organisms. The observed up-regulated expressions of these genes indicate that an environmental stressor can significantly affect genes participating in stress responsiveness or detoxification pathways. Thus we confirmed that transcriptional changes in these six genes can be used as indicators of an organism's exposure to toxicants in general, oxidative stress, heavy metals, or reproductive toxicants.

Methods

Animal

Rockfish (300-350 g, less than 2 years, male) were

obtained from The Sunghae Fisheries Cooperative (Geoje, Korea) and acclimated for 2 weeks in an aquatic facility. Three groups of fishes were exposed to filtered seawater containing 1 μM of BaP [dissolved in DMSO] (Sigma) for 6, 12, and 24 hrs, respectively. Control fish group was maintained in filtered seawater containing 0.1% DMSO. 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 containing 0.1% DMSO were used as controls.

Target Genes Cloning

The isolated RNAs were confirmed its sufficient quality for cDNA synthesis by spectrophotometric method and the ratios of the absorbance at 260 nm and 280 nm were ranged from 1.6 to 1.8 and those of at 230 nm and 260 nm were ranged from 1.8 to 2.0. To synthesize first strand cDNA, 2 μg of total RNA was extracted from 24 hrs BaP exposed rockfish liver and reverse transcribed with oligo d (T) primer using a Reverse Transcription System (Promega). Amplification was carried out in a thermal cycler (Takara, Japan) by denaturing for 5 min at 95°C, followed by 30 amplification cycles of 30 sec at 95°C, 30 sec at 56°C, 30 sec at 72°C, and a final extension for 7 min at 72°C. Secondary PCR with nested primers, as indicated in Table 1, was performed for GST, MT, UB, and VTG amplification, using the same conditions as used for primary PCR. The PCR products were ligated into a pGEM-Teasy vector (Promega) and the plasmids containing PCR products were isolated from the selected transformants by ampicillin and X-gal screening. Purified DNAs were sequenced using an ABI 3100 DNA Sequencing System (Applied Biosystems Inc.) with T7 and SP6 primers.

Northern Blot Analysis

Total RNAs (20 μg) from 4 groups (non-exposed control, 6 hrs, 12 hrs, and 24 hrs BaP-exposed fishes) were fractionated on 1.2% formaldehyde-agarose gel and blotted onto Hybond N^+ nylon membrane (Amersham). The cDNA fragments of the six gene clones were labeled with [α-^{32}P]dCTP (Amersham) using a random primed labeling kit (Roche). Hybridization and washing were carried out as recommended by the manufacturer of rapid hybridization buffer (Amersham Bioscience). The membrane was exposed for 3 hrs to X-ray film (Agfa) and developed. The northern blot image was analyzed using NIH Scion Image System (www.scioncorp.com). All data were presented as mean ± S.D. Two-group comparisons of control and treated fish were done by Students *t*-Test. Statistical significance was assigned at $P < 0.05$.

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