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Analysis of Gene Expression in Benzo[a]pyrene-exposed *Sebastes schlegeli* using Differential Display Polymerase Chain Reaction

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DD-PCR을 이용한 벤조피렌 노출 조피볼락의 차등 발현 유전자 분석

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요 약

오염물질의 노출에 의해 발현이 변화되는 유전자의 발굴은 외부환경 자극에 대한 적응이나 반응의 메커니즘을 알아내는데 중요한 정보를 제공하며, 오염물질에 반응하는 유전자는 환경오염을 감지하는 분자마커로 개발될 수 있다. DD-PCR 기법은 차등 발현 유전자들을 발굴해내기 위한 유용한 방법으로 사용되어 왔고, 본 연구는 이 방법을 이용하여 벤조피렌에 반응하는 조피볼락 유전자들의 발굴을 목적으로 진행되었다. 간조직에서 추출한 RNA로부터 벤조피렌의 노출에 의해 발현 양이 달라진 12개의 클론을 발굴하였고, 그 염기서열을 분석하였다. 또한 벤조피렌의 노출시간을 각각 6, 12, 24시간으로 달리한 조피볼락에서 12개의 클론 중 4개의 클론에 대해 northern blot 분석이 실시되었으며, 이들 모두 노출시간에 따라 발현양이 증가 또는 감소하는 것이 확인되었다. 본 연구결과는 오염물질의 영향에 의한 유전자들의 발현에 관한 전반적인 지식을 제공하였고, 나아가 환경오염이나 외부 스트레스를 감지해 낼 수 있는 바이오마커의 개발을 위한 첫 단계로서의 정보를 제공할 수 있을 것으로 생각된다.

Key words : *Sebastes schlegeli*, benzo[a]pyrene, DD-PCR, differential gene expression, biomarker

INTRODUCTION

The representative environmental stress in marine ecosystem is anthropogenic contamination such as

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sewage containing persistent organic pollutants and a variety of toxic chemicals from land runoff. In coastal area extrinsic endocrine disrupting materials such as polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides were widely distributed. According to Koh *et al.* (2001), the concentration of chlorinated compounds such as PCBs and dichlorodiphenyl trichloroethanes (DDTs) among the organic pollutants in Korean coastal environment was relatively lower than those in other developed countries. However, the record on PAHs which are toxic compounds and derived from oil spills by ship accident showed a higher concentration in Korean coastal regions (Koh *et al.*, 2001). Benzo[a]pyrene (BaP), a priority PAHs, is a representative ecotoxicant and has been reported bioaccumulative potential in many organisms resulting in DNA damage, endocrine disruption, and reproductive disturbance (Warshawsky *et al.*, 1995; Kepley *et al.*, 2003). 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.

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

Differential display PCR (DD-PCR) is a technique in which mRNA expressed by a cell population are reverse transcribed and then amplified by many separate polymerase chain reactions (Liang and Pardee, 1992). 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. The relatively simple procedure allows identification of genes that are differentially expressed in different cell populations. In this study, we aimed to explore the toxicant-responsive genes in rockfish, *Sebastes schlegeli*, which is a relatively abundant representative coastal species in the South Sea, using DD-PCR technique and to obtain the molecular-level information investigating the changes of gene expressions in fish affected by exposure to BaP.

MATERIALS AND METHODS

1. Animal

Rockfish (300~350 g) were obtained from Sunghae Fisheries Cooperative (Geoje, Korea) and acclimated for 2 weeks in an aquatic facility. Nine fish were assigned to filtered seawater containing 2 μM of BaP [dissolved in DMSO] (Sigma) and three control fish were maintained in filtered sea water containing 0.1% DMSO. Three fish were taken for RNA extraction from the BaP-treated seawater tank at 6, 12, and 24 hr, respectively. 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 control.

2. Differential display (DD-) PCR and gene cloning

The first strand cDNA was synthesized by using moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega, WI, USA) following the manufacturer's direction. The total RNA was used as a template and primed with oligo $(dT)_{15}$-ACP (Seegene, Seoul, Korea, Kim *et al.*, 2004). Differential display PCR was performed by using GeneFishing DEG kit (Seegene, Seoul, Korea, Kim *et al.*, 2004) to identify differentially expressed genes. Manufacturer's direction for ACP-based GeneFishing-PCR was followed. Forty arbitrary ACP primers (A1-20, A41

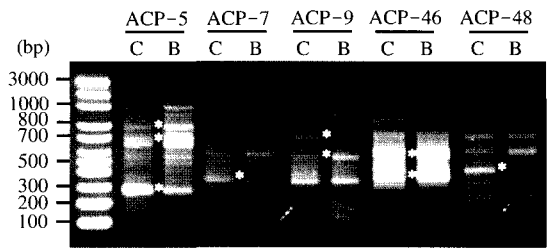
-60) were applied to the PCR. The PCR products were separated in 2% agarose gel stained with EtBr. The differentially expressed PCR products were cut out from the gel and extracted by using QIAquick gel extraction kit (Qiagen). The purified PCR products were cloned into the pGEM-T Easy vector (Promega). Sequencing of positive clones was carried out with an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

3. Northern blot analysis

Total RNAs (20 μg) from 4 groups (non-exposed control, 6, 12, and 24 hr BaP-exposed fishes) were fractionated on 1.2% formaldehyde-agarose gel and blotted onto Hybond N^+ nylon membrane (Amersham Bioscience). The cDNA fragments of the four gene clones were labeled with [α-^{32}P]dCTP (Amersham Bioscience) 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 hr to X-ray film (Agfa) and developed. The northern blot image was analyzed using NIH Scion Image System (www.scioncorp.com).

RESULTS AND DISCUSSION

DD-PCR has been developed to identify and isolate differentially expressed genes (Liang and Pardee, 1992) and extensively applied to various range of differential gene expression analyses because of its effectiveness and convenience. One of the merits of this technique is requirement of only small amount of RNA since this technique is PCR-based. However, relatively high chance to have false-positive acts as a major handicap in this technique. Many efforts have been attempted to improve the specificity of DD-PCR (Liang *et al.*, 1993; Liang *et al.*, 1994). Recently epochal modification to eliminate false-positive has been developed by increasing the annealing specificity with specially designed annealing control primer (ACP) system (Hwang *et al.*, 2003; Kim *et al.*, 2004). In this study, we applied the



C: Control
B: Benzo[a]pyrene exposed (24 h)

Fig. 1. Differential display of mRNA from *Sebastes schlegeli* exposed to benzo[a]pyrene. C, 0.1% DMSO exposed control; B, benzo[a]pyrene exposed. The candidates of differentially expressed genes were indicated by asterisk (*).

Table 1. Differentially expressed genes with known function identified by DD-PCR.

Clone	Size (bp)	Putative identity[a]	DD-PCR[b]
SS4-400	245	Allatotropin neuropeptide precursor	Up
SS4-500	493	Alpha-interferon inducible protein	Up
SS4-500	395	Penicillin G acylase precursor	Up
SS4-500	467	Gluconolactonase	Up
SS5-400	474	Fructose-1,6-bisphosphate aldolase	Up
SS5-700	342	Betaine-homocysteine methyltransferase	Down
SS5-800	472	Warm-temperature-acclimation-related-65 kDa-protein-like-protein	Up
SS5-900	749	BCL2/adenovirus E1B 19 kDa-interacting protein	Up
SS9-380	309	Glycine max beta-carotene hydroxylase	Up
SS15-550	346	Translation initiation factor eIF-2B beta subunit gene	Up
SS43-380	353	Serum amyloid A protein	Up
SS48-320	216	Cytochrome P450 1A	Up

[a]Basic Local Alignment Search (BlastX) at NCBI

[b]Up- or down-regulated in benzo[a]pyrene exposed rockfish (24 hr)

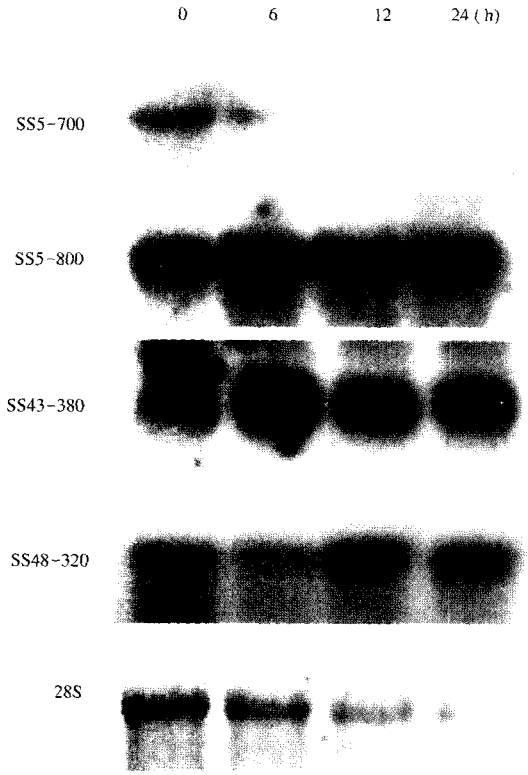


Fig. 2. Northern blot analyses of the differentially expressed DD-PCR products in different duration of benzo[a]pyrene exposure. Four kinds of RNA from 0, 6, 12, and 24 h of benzo[a]pyrene exposed animal were used to evaluate the different expression level of candidate genes which have identified by DD-PCR. For internal control, 28S ribosomal RNA was used.

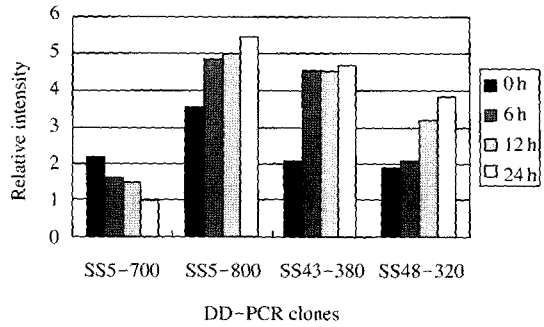


Fig. 3. Quantitative comparison of gene expression levels obtained from northern blot analyses. Densitometric analysis of northern blot bands was performed using NIH Scion Image Analysis (www.scioncorp.com). The intensity values from 0, 6, 12, and 24 hr of benzo[a]pyrene exposure samples were normalized using the intensity value of 28S ribosomal RNA as internal control, respectively.

ACP system to identify the differentially expressed genes responding to the chemical contaminant such as benzo[a]pyrene (Fig. 1). The GeneFishing kit, a commercial brand based on ACP system, has two additional merits, which derived from the high specificity of primers. First is, PCR products could be separated on agarose gel because the improved specificity and sensitivity result only a few amplified product. Second is, no usage of radio isotope. Non-radioactive detection of the products increases convenience and facilities.

This study aimed to discover the genes which transcriptions were changed by the environmental toxicant benzo[a]pyrene 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 step, we obtained 12 fragments whose expressions altered by BaP exposure comparing with those of unexposed control using DD-PCR technique (Table 1). Of the 12 fragments, four clones were analyzed by northern blot to confirm their expression changes by BaP exposure duration (Fig. 2). The result displayed significant change of gene expression in accordance with BaP-exposure time in densitometric analysis of northern blot (Fig. 3). 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 1.5 and 2.2-fold at 24 hr post-BaP exposure, compared with 0 hr control.

Clone SS5-700, which nucleotide sequence showed homology to betaine homocysteine methyltransferase in *Mus musculus* (DDBJ/EMBL/GenBank accession No. BC013515) was repressed gradually from 6 hr post-exposure and 2.2-fold downregulated at 24 hr post-exposure compared with 0 hr control. According to the recent report, betaine homocysteine methyl-

transferase (BHMT) was identified as a gene down-regulated in rat liver cirrhosis and most likely involved in dysregulation of homocystein level (Forestier *et al.*, 2003; Jalan *et al.*, 2003). 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 upon the molecular mechanisms involved in the pathogenesis of hepatic encephalopathy with liver dysfunction incorporating with BHMT and therapy of hepatic encephalopathy to reduce ammonia generation and increase its detoxification (Jalan *et al.*, 2003).

Clone SS5-800 was identified homologous to *Oryzias latipes* warm-temperature-acclimation-related 65 kDa protein-like-protein (AB075199). Its expression was increased at 6 hr post-BaP exposure with 1.3-fold expression change and showed 1.5-fold upregulation 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 (WAP) (Picard and Schulte, 2004). Those genes significantly changed in expression between populations from different thermal environments and especially expression of WAP was eight-fold higher in Southern than in Northern fish, consistent with a previously suggested role for this gene in thermal acclimation or adaptation to extrinsic stresses in fish (Picard and Schulte, 2004). 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.

Another clone, SS43-380 showing 2.2-fold higher expression in 24 hr BaP exposure, had homology to *Holothuria glaberrima* serum amyloid A protein (AF300706). Comparing with 0 hr control, its expression from 6 to 24 hr post-BaP exposure was drastically increased. Serum amyloid A is known as a superfamily of acute-phase proteins. The level of serum amyloid A in blood increases dramatically in response to tissue injury and inflammation, influencing cell adhesion, migration, proliferation and aggregation (Lycopoulou *et al.*, 2005). In human, serum amyloid A 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 (Guo *et al.*, 2002). 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 (Fournier *et al.*, 2002). 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 (Anderson *et al.*, 1997).

The last clone named SS48-320, which level of expression was proportionally upregulated after BaP exposure, showed high similarity to *Dicentrarchus labrax* cytochrome P450 1A (U78316). CYP1 gene family is efficient metabolizers of PAHs and was induced in fish by various PAHs (Stegeman and Kloepper-Sams, 1987). 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 (Gonzalez and Kimura, 2003). CYP1A protein was found to be significantly higher at contaminated sites in response to PAHs and PCBs in the mussel *Mytilus* sp. (Shaw *et al.*, 2004), in the sole *Pleuronectes vetulus* (Miller *et al.*, 2004), and in the salmon *Salmo salar* (Boon *et al.*, 2002).

In conclusion, we successfully isolate four genes

differentially expressed in response to the existence of BaP and the exposure duration. 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 known to be related to pathogenesis, extrinsic stresses, and catalytic metabolites. Considering the expected function of the isolated genes, the ACP-based DD-PCR method 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 environmental stress or contamination.

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