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Toxicity evaluation of benzo[*a*]pyrene on the polychaete *Perinereis nuntia* using subtractive cDNA libraries

Senlin Zheng[a,*], Xiaoyan Qiu[b,1], Bin Chen[a,**], Xingguang Yu[a], Kangli Lin[a], Mei Bian[a], Zhenghua Liu[a], Hao Huang[a], Weiwei Yu[a]

[a] *Third Institute of Oceanography, State Oceanic Administration, Xiamen, 361005, China*
[b] *College of Bioengineering, Jimei University, Xiamen, 361021, China*

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ABSTRACT

To gain insight into the toxic effects of the carcinogenic PAH benzo[*a*]pyrene (BaP) on the typical marine benthic polychaete *Perinereis nuntia*, we amplified and sequenced genes by creating subtractive cDNA libraries between worms exposed to BaP and solvent control. We assigned functions to the identified sequences and further analyzed the transcriptional profile changes of a set of 50 selected potential marker genes using quantitative real time PCR. A total of 2422 new high quality ESTs (GenBank accession number GT629654–GT632075) were obtained in the *P. nuntia* subtracted cDNA libraries, and assembled into 1594 unique sequences. Blastx results showed 700 of the unique sequences shared high similarity with existing genes in the GenBank nr database. Functional annotation of these enriched gene segments suggested that *P. nuntia* shows a wide range of toxicological responses to BaP. Comparison of the transcriptional profiles of the 50 potential marker genes in worms exposed to BaP and the control suggested that BaP significantly changed the expression of genes involved in xenobiotics metabolism, reactive oxygen species (ROS) elimination, DNA repair, apoptosis, cell division cycle, neurodegeneration, neurotransmitter metabolism and carcinogenesis. It also shows that there are significant correlations between these potential marker genes. The results support the prediction that the polychaete *P. nuntia* also has a set of tumor-related genes, while other responses influenced by BaP involve detoxification, antioxidation, DNA repair and apoptosis.

1. Introduction

Marine environmental pollution has raised considerable concern given that some pollutants can be genotoxic, carcinogenic, and teratogenic (De Flora et al., 1991). One such pollutant, benzo[*a*]pyrene (BaP), is a carcinogenic and mutagenic polycyclic aromatic hydrocarbon (PAH), which mainly comes from petroleum and its refined products, and the incomplete combustion of coal, petroleum, and biomass (Zheng et al., 2004). Many hydrophobic organic pollutants including PAHs that enter aquatic environments become adsorbed onto the surface of particles within the water. These particles then precipitate from the water and become part of the sediment (Simkiss et al., 2000). Benthic organisms are often used as sentinels for assessment of sediment contamination (Hagger et al., 2008).

Polychaeta are a large class in phylum annelid. Worldwide there are known to be over 10,000 species of polychaeta living in locations ranging from tideland to deep sea (Metcalfe and Glasby, 2008). Polychaeta play an important role in the marine ecosystem as detritivores, feeding on the sea floor, they are also prey for larger fish, snails and other predators. Polychaeta are often used as an index to qualify the degradation status of marine coastal areas (Canfield et al., 1996; Afli et al., 2008; Zheng et al., 2010). However, knowledge of the effects of environmental carcinogens on polychaeta is very limited. *Perinereis nuntia* is one of the most widespread polychaeta in shore line habitat and is frequently used as indicators of environmental contamination.

Abbreviations: BaP, benzo[*a*]pyrene; Blastx, basic local alignment search tool; BP, biological process; CC, cellular component; CDC, cell division clycle; CYP, cytochrome P450; DMSO, dimethyl sulfoxide; EST, expressed sequence tag; GO, gene ontology; KEGG, kyoto Encyclopedia of Genes and Genomes; MAFK, V-maf musculoaponeurotic fibrosarcoma oncogene homolog K; MF, molecular function; PAH, polycyclic aromatic hydrocarbon; PDCD10, programmed cell death 10; ROS, reactive oxygen species; SSH, suppression subtractive hybridization; RT-q-PCR, reverse transcription and quantitative PCR.

* Corresponding author at: 184 Daxue Road, Xiamen, 361005, China. Tel.: +86 592 2195313, fax: +86 592 2191929.
** Co-corresponding author.
E-mail addresses: zhengsenlin@yahoo.com.cn, zhengsenlin@msn.com (S. Zheng).
[1] Co-first author.

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Biomarkers were originally defined as any biochemical, histological, or physiological alterations or manifestations of environmental stress. More recently, biomarkers were redefined as biochemical sublethal changes resulting from an individual exposure to xenobiotics (Hyne and Maher, 2003). Biomarkers have been used extensively as indicators of biological responses in laboratory studies in relation to individual contaminants or stressors. They are also frequently used in field surveys to assess the general health of organisms inhabiting contaminated ecosystems (Hagger et al., 2008). A number of invertebrate biomarkers for toxicity assessment have been identified and applied in the field to test the quality of waterway sediments for the past decade (Snyder, 2000; Amiard et al., 2006; Matozzo et al., 2008), however little has been discovered about the marker genes in the marine polychaeta when exposed to carcinogenic xenobiotics.

The development of genomics has provided a powerful tool for many disciplines of research, and genomic technology has been incorporated into ecological studies and developed a new discipline, ecotoxicogenomics (Snape et al., 2004). Ecotoxicogenomic techniques provide holistic tools for ecotoxicology studies in many areas such as chemical screening, environmental monitoring and risk assessment, and these tools may be more informative than traditional toxicity assessment methods (Poynton et al., 2008). Unfortunately, limited or non-existing sequence data of relevant organisms as well as other challenges are currently delaying the growth of ecotoxicogenomics and make the interpretation of results difficult (Poynton et al., 2008). However, suppression subtractive hybridization PCR (SSH-PCR), developed by Diatchenko et al. (1996), is a tool that can be applied to study the holistic change of genes expression in non model organisms. This method has previously been successfully applied in the ecotoxicological study of earthworms (Pirooznia et al., 2007).

To identify the impact of the carcinogen BaP on transcription in the marine polychaete, this study has presented sequencing results of the forward and reverse subtractive cDNA libraries constructed by SSH-PCR in a widespread polychaete *P. nuntia* following BaP exposure. This study also analyzes the potential functions of proposed marker gene identified in the enriched cDNA clones using Blastx searches, gene ontology (GO) annotation, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping and quantitative real time PCR.

2. Materials and methods

2.1. Animals and exposure experiment

Adult sandworm *P. nuntia*, 400–500 mg wet weight, about two month before turning into heteronereis stage, purchased from a bait farm in Fuzhou, China, were sampled and acclimatized in clean filtered seawater with 10 cm pebble bed in glass tanks (50 cm × 40 cm × 40 cm) for 1 week.

BaP (analytical purity, CAS number 50-32-8, Fluka, Switzerland) dissolved in DMSO was used to artificially contaminate seawater to a final concentration of 0.01 mg L^{-1}. An equivalent amount of DMSO was added to filtered clean seawater as solvent control. Groups of 20 sandworms were randomly assigned to seawater with about 10 μg L^{-1} BaP and the solvent control group. After being cultured under laboratory conditions (20 ± 1 °C, salinity 30‰, 12 h dark:12 h light, DO ≥ 6.0 mg L^{-1}) for 96 h, sandworms in both the treated and control groups all survived and did not show any observable differences. They were collected and stored in liquid nitrogen for further analysis. Using the national standard method of China (GB 13198-91), the concentration of BaP was determined to be 9.63 μg L^{-1} in the artificial pollution group after 96 h exposure, and undetected in the solvent control.

2.2. RNA extraction and mRNA purification

Polychaeta of similar wet weight (440 ± 10 mg) from the BaP-exposed group and also the solvent control were individually ground into powder in a ceramic mortar with liquid nitrogen. Total RNA of each whole worm was isolated following the RNAiso Reagent (Takara Biotech., Dalian, China) protocol. The RNA purity and integrity were checked by ensuring that absorbance ratios (A260/280) were between 1.8 and 2.0 and by agarose gel (1.5%) electrophoresis.

The mRNA of each sandworm was purified with the Neucleo Trap® mRNA purification Kit (Clontech, USA) and concentrations of poly A^+ RNA were measured using a Unicam UV-300 Spectrophotometer (Thermo Spectronic, USA).

2.3. cDNA subtractive libraries construction

Using a Clontech PCR-Select™ cDNA subtraction kit (Clontech, USA), forward subtraction, reverse subtraction and the control subtraction experiments were carried out in parallel. For the forward subtraction, the cDNA synthesized from the worm exposed to BaP was used as the tester, and the cDNA from the worms of the solvent control were used as the driver, to enrich the upregulated sequences activated by BaP. For the reverse subtraction, the cDNA of the solvent control worm was used as the tester, and the cDNA of the BaP-exposed worms were used as the driver, to enrich the downregulated sequences inhibited by BaP. The same amount of poly A^+ RNA (1.2 μg) from each whole sandworm sample treated with or without BaP as well as the control skeletal muscle mRNA were used to synthesized cDNA. Rsa I digestion of the synthesized cDNA, adaptors ligation, and two steps of hybridization, as well as and the efficiency tests were operated strictly following the protocol of the subtraction kit. The last two steps of suppressive PCR reactions were completed using a BD advanced cDNA PCR kit (BD, USA). The amplified subtractive *P. nuntia* cDNA was purified with a DNA Fragment Purification Kit (Takara Biotech., Dalian, China) and cloned into the PMD 18-T vector (Takara Biotech., Dalian, China), then transferred into DH5α competent cells. The transformed cells were transferred onto solid LB media added with 80 μg mL^{-1} ampicillin, and incubated at 37 °C for about 16 h until white colonies grew. Each white clone was picked separately and cultured in 5 mL LB liquid media at 37 °C for 6 h. Using the nested primers of the adaptors supplied in the cDNA subtraction kit and the transformed clones as a template, the insert cDNA were amplified and the sizes were analyzed by agarose gel (1.5%) electrophoresis. The clones that revealed single bands on the gel were sequenced and further analyzed.

2.4. Sequencing, clustering and Blastx

Each clone was sequenced using an AB3730 DNA sequencer (AB, USA). For each good quality sequencing result, the vector and adaptors were cleaned and then the insert was used for the next step of analysis. Each insert sequence with the assigned clone number was listed in a fasta format file. To identify repeats to overlay the sequences, we formatted all the sequencing results into a database and compared each EST against the database with the downloaded Blast tool. Each primarily assembled sequence was compared to the downloaded non-redundant protein (nr) databases using the downloaded Blastx tool with the *E*-value cut-off set to 1E−5 to identify putative functions of the ESTs (Pirooznia et al., 2007). The sequences with the same Blastx hits were further assembled if they shared adjacent amino acid sequences of the same existing protein. Using this method we were able to identify 1594 unique *P. nuntia* sequences and assigned them unique sequence ID.