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Digital gene expression analysis of reproductive toxicity of benzo[a]pyrene in male scallop *chlamys farreri*

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ABSTRACT

Benzo[a]pyrene (BaP) is a representative polycyclic aromatic hydrocarbon (PAH) and is studied widely for its strong toxicity and wide distribution. Although BaP pollution in marine environment is increasing, molecular mechanisms underlying reproductive toxicity of BaP in marine mollusks have been seldom systematically studied, especially in males. In this study, genes that regulated reproductive responses of *Chlamys farreri* under BaP stress were analyzed through digital gene expression (DGE) sequencing with testis tissues. A total of 12,485,055 and 14,454,127 clean reads were generated from control and BaP exposure DGE libraries, respectively. After comparing two libraries, 1051 differentially expressed genes were detected, with 223 up-regulated and 828 down-regulated genes. Gene ontology (GO) annotation and kyoto encyclopedia of genes and genomes (KEGG) pathway analyses were performed on all genes to understand their biological functions and processes. The results showed that numerous enriched, differentially expressed genes related to aromatic compound catabolic processes, spermatid development, microtubule-based movement, energy production and immune response. Quantitative real-time PCR was performed to verify the expressed genes of DGE. The study generated data to show the overall reproductive transcription responses of male *C. farreri* under BaP stress, and it also can serve as the reference for future study of organic pollutions in aquatic mollusks.

1. Introduction

With the growing environmental pollution, exogenous chemicals have posed threats to the reproductive health of various species, which made biological reproductive toxicity become a global concern. Numerous studies have reported the changes of reproductive performances in different organisms, for instance, Oberdõrster and McClellan-Green, 2000 reported that TBT and TPT could induce sexual aberrations and sex reversal in mud snails; DDT exposure generated degenerating oocytes in the zebra mussel (*Dreissena polymorpha*) (Binelli et al., 2004) and Benzo[a]pyrene (BaP) resulted in lower levels of free sex hormones in *Sebastiscus marmoratus* (Zheng et al., 2006). BaP, as the representative carcinogenic polycyclic aromatic hydrocarbon (PAH), is a ubiquitous environmental pollutant that is currently regarded as an endocrine disruptor (Chung et al., 2011; Sadeu and Foster, 2011). The United States, Japan and the European Union had implemented endocrine disrupting chemicals (EDCs) screening programs including screens for BaP (Chen et al., 2011). According to the cumulative data, BaP is widely distributed in Chinese coastal water and the concentration is quite different, ranging from 0.16 to 663 ng/L (Qiu et al., 2004). The average BaP content in marine sediments is much higher (Huang et al., 2012a, 2012b; Liu et al., 2012a, 2012b).

The reproductive toxicity of BaP in previous studies mainly focused its damage effects and endocrine disrupting effects on vertebrate, including that BaP could cause DNA strand breaks, lipid peroxidation and protein carbonylation (Frenzilli et al., 2004; Regoli et al., 2004); disturb expressions of reproductive-related genes via estrogen receptor-aromatic hydrocarbons receptor (ER-AHR) cross-talk mechanism (Tsai et al., 2004; Gräns et al., 2010; Matthews and Ahmed, 2013) as well as alter gene expression of critical steroidogenic enzymes for the production of estradiol (Rocha Monteiro et al., 2000; Zheng et al., 2006). Siah et al. (2003) reported that marine invertebrates are also sensitive to endocrine disruptors at environmentally relevant concentrations. However, due to technical limitations, investigations on reproductive toxicity mechanisms of BaP in marine invertebrates were insufficient, especially in males. The testis was reported an important target for BaP (Archibong et al., 2002), but its underlying molecular mechanisms in marine invertebrates were still unclear.

RNA sequencing technologie (RNA-seq) represents clear advantages over existing approaches with sequencing depth and sequencing accuracy, which can supply reliable measurements of transcript levels in various conditions (Ekblom and Galindo, 2011; Wang et al., 2009). Specifically it can provide valid and rich

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experiment data towards specific transcriptome information. Digital gene expression (DGE), a tag-based transcriptome sequencing method for measuring relative gene expression levels on the basis of RNA-seq, thus becomes a very appropriate technology to analyze the full transcriptional changes caused by reproductive toxicity of BaP.

Bivalve mollusks are widely used as biological indicators of marine pollution because of their feature of pollutant bioconcentration and filter-feeding habits (Liu et al., 2012a, 2012b; Goldberg et al., 1978). Among bivalve mollusks, the scallop *Chlamys farreri* is a kind of important aquaculture bivalve of high commercial and ecological value in coastal areas of northern China (Pan et al., 2008). Adding the characteristics of specific reproductive cycle and gonochorism make them suitable model to explore the reproductive effects under BaP exposure. In this study, two DGE libraries (control and BaP exposure) were constructed from the testis of *C. farreri* to examine the reproductive transcription response to BaP stress. The study aims to provide a more complete evaluation of BaP toxicity on the reproductive organ of male *C. farreri* from the molecular level and screen the molecular biomarkers of marine environment under PAHs pollution. This study provided important data resources for the PAHs pollution monitoring of marine environment and the germplasm resources protection of bivalves.

2. Materials and methods

2.1. Animals and BaP treatment

In this study, any work involving humans or experimental animals were conducted in accordance with national and institutional guidelines for the protection of human subjects and animal welfare. Adult scallops (*C. farreri*) in sex mature stage were purchased from Taiping Bay (Yellow Sea, Qingdao, China) on June 26th, 2013 with the average shell length 7.2 ± 0.5 cm. After purchased, they were acclimated in polystyrene tanks (50 cm × 40 cm × 30 cm) containing aerated sand-filtered seawater (salinity 31‰, pH 8.1, temperature 20 ± 1 °C) and aerated continuously for two weeks before the exposure experiment. During the acclimatization, the water in each tank was renewed completely once a day and the scallops were fed with dried powder of *Spirulina platensis* (3 g/cm^3 water per day).

For DGE analyses, one group of *C. farreri* was exposed to BaP at the concentration of 0.5 μg/L and another group of *C. farreri* without any additional pollutants was set as control. BaP was dissolved in dimethylsulfoxide (DMSO) at its final concentration of 0.001 percent. The exposure process lasted for ten days and all the experimental conditions were the same as acclimatization. Three replicates were set for treatment group and control group respectively. No mortalities were observed during the exposure experiment. At day 10, six male scallop gonads of each replicate were excised and grinded in liquid nitrogen immediately and kept at −80 °C.

2.2. RNA extraction

Two samples (control and BaP treatment) of male scallop gonads were added with 1 ml of TRIzol reagent (Invitrogen) respectively to extract the total RNA in accordance with manufacturer's instructions. Total RNA degradation and contamination were monitored on one percent agarose gels. RNA concentration was measured using Qubit RNA Assay Kit in Qubit 2.0 Flurometer (Life Technologies, CA, USA) and its integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA).

2.3. cDNA library preparation and sequencing

Sequencing libraries were generated using NEBNext Ultra™ RNA Library Prep Kit for Illumina (NEB, USA) following the manufacturer's recommendations. A total amount of 3 μg RNA per sample was used to purify mRNA using poly-T oligo-attached magnetic beads and then fragment mRNA into short fragments. First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase (RNase H$^-$). Second strand cDNA synthesis was subsequently performed using DNA polymerase I and RNase H. After adenylation of 3' ends of DNA fragments, NEBNext Adapter was ligated to prepare for hybridization. cDNA fragments of 150–200 bp in length were selected for PCR amplification to create a cDNA library. The library preparations were sequenced on an Illumina Hiseq2000 platform and generated 100 bp paired-end reads.

2.4. Data analysis

Raw data sequenced from the last step was saved as fastq format. Raw reads containing adapter, ploy-N and low quality reads were filtered to obtain high quality clean data. The high quality clean reads were mapped using Bowtie v0.12.9 to the unigene of *C. farreri* transcriptome datasets (SRP018044) sequenced by the laboratory previous work. More than 1 bp of mismatch was not considered. Clean tags mapped to the reference sequences from multiple genes were filtered. The remaining clean tags were designated as unambiguous clean tags. The number of unambiguous clean tags mapped to each gene was counted by RSEM and transformed into RPKM (Reads per Kilo bases per Million reads) (Mortazavi et al., 2008).

2.5. Differential gene expression analysis

Prior to differential gene expression analysis, the read counts of two sequenced libraries were first adjusted by edgeR program package through one scaling normalized factor. Then the two cDNA libraries were performed using the DEGSeq R package (1.12.0) to detect gene expression changes. The *p*-values were adjusted using the Benjamini and Hochberg method (Benjamini and Hochberg, 1995). Corrected *p*-value (*q*-value) < 0.005 and $|\log2 \text{ (Fold change)}| >$ one were set as the threshold for significantly differential expression.

2.6. GO and KEGG

GO enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias were corrected. GO terms with *P* value < 0.05 were considered significantly enriched of the gene sets. KEGG database was used to excavate high-level functions and utilities of the biological system of differentially expressed genes and identify significantly enriched metabolic pathways or signal transduction pathways. The statistical enrichment of differential expression genes in KEGG pathways of this study were tested by KOBAS software (2.0). Pathways with *P* value < 0.05 were considered as significantly enriched items.

2.7. qRT-PCR analysis

Two samples (control and 0.5 μg/L BaP treatment) with the same as previous DGE samples were selected to execute the quantitative RT-PCR (qRT-PCR) study. Total RNA was extracted utilizing RNAiso Plus (TaKaRa Corp., Dalian, China) with genomic DNA eliminated by RNAse-free DNase. Then 2–4 μg of total RNA was applied to synthesize the first strand cDNA with PrimeScript Reverse Transcriptase kit (TaKaRa) following the manufacture's protocol. Eight genes related to detoxification of BaP within *C. farreri* and testicular developments were performed by qRT-PCR. β-actin of *C. farreri* was selected as the reference gene. All the gene primers were shown in Table S1. qRT-PCR was implemented in a PikoREAL96 Real-Time PCR System (Thermo Scientific., America) using SYBR Premix Ex TaqTM kit (TaKaRa). The real-time RT-PCR program was 95 °C for 30 s, then 40 cycles of 95 °C for 10 s, 55 °C for 20 s and 72 °C for 30 s. The relative expression level of target genes was expressed in sample versus control in comparison to the reference gene (Pfaffl., 2001). Results are shown as mean ± standard deviation of three replicates.

3. Results

3.1. DGE libraries and mapping analysis

To investigate the comprehensive transcriptional mechanisms of reproductive toxicity of BaP in scallop *C. farreri*, two DGE libraries were constructed from testis of control and BaP exposure applying Illumina technology. A total of 12,686,988 and 14,693,487 raw reads were generated from control and BaP exposure DGE libraries respectively, with 12,485,055 and 14,454,127 clean reads left for assembly after removing low quality reads (Table 1). The data had already been uploaded to the SRA database (accession number: SRP034888). Then all clean tags were aligned to the reference *C. farreri* transcriptome database (SRP018044) which contained 92,999 unigenes. Among two DGE libraries, approximately 74.24 percent and 77.27 percent of the clean tags could map to the reference sequences (Table 1). In this study, RPKM, reads per kilobase of exon model per million mapped reads considering the effect of sequencing depth and gene length for the reads count at the same time, was used to calculate gene expression levels (Mortazavi et al., 2008).