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Identification and differential expression of hepatopancreas microRNAs in red swamp crayfish fed with emodin diet

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ABSTRACT

Using high-throughput Illumina Solexa system, the differential miRNA expressions from hepatopancreas in red swamp crayfish (*Procambarus clarkii*) fed with diets containing 0 (control) and 75 mg emodin kg^{-1} (trial) were identified, respectively. As a result, 13,335,928 raw reads from the control sample and 14,938,951 raw reads from the trial sample were obtained while 13,053,344 (98.77%) and 14,517,522 (98.34%) small RNA were identified, respectively. 106 mature miRNAs (belonging to 68 miRNA gene families) were identified. 35 miRNAs displayed significantly differential expressions between two libraries. Of these, comparing to the control library, 6 miRNAs were significantly up-regulated and 29 miRNAs were significantly down-regulated. Moreover, 5 novel miRNAs (2 from control sample, 3 from trial sample) and target genes were predicted. GO analysis suggested that these miRNAs might be involved in innate immune response, growth, metabolism, cellular process, biological regulation and stimulus response. Our knowledge from this study could contribute to a better understanding of the miRNAs roles in regulating innate immune response and the study of miRNA function in crayfish.

1. Introduction

Red swamp crayfish (*Procambarus clarkii*) was native to the southeastern United States and had been introduced worldwide. It was the most common freshwater crayfish specie in China now. However, with the development of intensive culture, water pollution, environmental degradation and frequent occurrence of various diseases resulted in economic losses of aquaculture [1]. Like other crustanceans, crayfish lacked an adaptive immune system and depended exclusively on the innate immune system to defend against potential pathogenic bacteria, viruses, and parasites. Therefore, in recent years, appropriate dietary immunoenhancers such as probiotics, functional sugars, were widely used in aquaculture to improve shrimp and crayfish innate immune system.

Emodin (1, 3, 8-trihydroxy-6-methyl-anthraquinone), a medicinal extract from herbs including rhubarb (*Rheum officinale Baill*), aloe (*Aloe barbadensis Miller*), senna (*Cassia angustifolia*), and thunberg (*Polygonum multiflorum*), had been widely used as a traditional medicine in Eastern Asia, especially in China [2]. Previous reports showed that emodin played a roles in antibacterial and anti-inflammatory [3], antioxidation and free radical scavenging [4], reduction of blood lipid concentration [5], hepatoprotection [6], and immune regulation [7]. In human medical studies, several mechanisms of emodin had been described as possible modes of emodin antitumor action. Firstly, emodin generated a reactive oxygen species, which resulted in cancer cells apoptosis. Secondly, emodin induced primary DNA lesions through alkylation of DNA, which led to the perturbation of the cell cycle. Finally, emodin inhibited specific kinase activities that were required for cancer survival [8].

Now, emodin as immunopotentiator had been studied and applied in fish and crayfish culture. The research indicated that the growth, non-specific immunity and high temperature tolerance of freshwater prawn (*Macrobrachium rosenbergii*) fed with diet containing emodin were improved significantly [9]. The studies on Wuchang bream (*Megalobrama amblycephala*) showed that appropriate dietary emodin supplementation (30 mg emodin kg^{-1} diet) could enhance the growth and immune responses and improve fish resistance to infection by *Aeromonas hydrophila* [1,10,11]. In our previous study, the growth, survival rate of crayfish fed with 75 mg emodin kg^{-1} diet were significantly increased. Serum lysozyme, ceruloplasmin and alkaline phosphatase activities were significantly improved. Significantly higher levels of

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hepatopancreas catalase, glutathione and superoxide dismutase activities were also observed [12]. Even so, the detailed mechanism which emodin mediated growth and immune function still remained to be determined.

MicroRNAs (miRNAs) were a large family of 21–22 nucleotide non-coding RNAs, which played very important roles in regulating gene expression by degradation of target mRNAs or by repression of targeted gene translation both in animals and plants [13]. Recently, more and more evidences suggested that miRNAs had diverse biological functions, such as embryo formation, organogenesis, cell death, cell proliferation, lipid metabolism and immune development [14–18]. Studies on crayfish microRNAs supported that certain miRNAs along with their target genes might be essential in the intricate host–pathogen interaction networks, and should help developing new control strategies for host immune defense against various foreign pathogens's infection in crustaceans [19].

In this paper, we investigated whether miRNAs involved in growth and immunoregulation of crayfish fed with diet containing emodin by using high-throughput Illumina Solexa analysis. This study could contribute to understanding of the miRNAs roles in regulating innate immune response and identification of emodin-associated miRNAs in crayfish.

2. Methods

2.1. Experimental animals and feeding trial

All animal experiments were performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China.

Formulation and proximate composition of the basal diet was presented in Table 1. The protein and lipid requirements for this species were set according to Xu et al. (2013) [1]. Fish meal, soybean meal, rapeseed meal and shrimp bran served as protein sources. Soybean oil was used as lipid sources. Wheat flour served as carbohydrate sources. Emodin (reagent grade, ≥98% (TLC), Xi'an Feida Bio-Tech Co., Ltd, Xian, China) was supplemented to the basal diet to formulate two diets containing 0 mg kg^{-1} and 75 mg kg^{-1} [12], respectively. All diets were prepared in the laboratory. Dry ingredients were ground through a 60-mm mesh. An appropriate amount of water was added to produce stiff dough. The dough was then pelleted using a laboratory pellet machine and dried in a ventilated oven at room temperature. After drying, the diets were broken up and sieved into proper pellet size. All diets were stored at −20 °C until used.

Table 1
Formulation and chemical composition of the basal diet.

Ingredient	% dry matter
Fish meal	5.00
Soybean meal	31.25
Rapeseed meal	16.00
Shrimp bran	3.00
Wheat flour	31.29
α-starch	4.00
Soybean oil	3.21
Attapulgite	1.00
Zeolite powder	1.5
Monocalcium phosphate	2.2
Salt	0.4
Premixture[a]	1.00
Chitin	0.15
Proximate composition (%)	
Crude protein	26.11
Crude lipid	5.83

[a] Premix provided the following minerals (g kg^{-1}) and vitamins (IU or mg kg^{-1}): $FeSO_4 \cdot 7H_2O$, 25 g; $ZnSO_4 \cdot 7H_2O$, 22 g; $MnSO_4 \cdot 4H_2O$, 7 g; Na_2SeO_3, 0.04 g; KI, 0.026 g; $CoCl_2 \cdot 6H_2O$, 0.1 g; Vitamin E, 15,000 mg; Vitamin K, 5000 mg; Vitamin B1, 8000 mg; Vitamin B2, 5000 mg; Vitamin B3, 15,000 mg; Vitamin B5, 15,000 mg; Vitamin B6, 5000 mg; Vitamin B7, 100 mg; Vitamin B12, 2 mg; Folic acid, 1000 mg; Vitamin C, 30,000 IU; Vitamin A, 1,000,000 IU; Vitamin D, 200,000 IU.

Healthy crayfish (average weight, 7.25 ± 0.24 g) were provided by freshwater fisheries research institute of Jiangsu province, China. During acclimation, the crayfish were fed three times daily to satiation. Thereafter, 360 crayfish were randomly distributed into 6 floating cages (3 × 2 × 1.5 m, L:W:H). Each cage contained 60 crayfish (female: male = 1:1). Each diet was tested in triplicate. During the 8-week feeding trial, the juvenile crayfish were fed with the respective test diet at a rate equaling 5% of body weight day^{-1}. Daily ration size was divided into three feedings at 0630, 1230, and 1830 h. Water temperature ranged from 26 to 30 °C, pH between 6.5 and 7.6, and dissolved oxygen (DO) was maintained at ~5.0 mg/l during the feeding trial. Temperature, DO, and pH were monitored twice daily (0600 and 1630 h) using a water quality meter (YSI 556MPS). Total ammonia nitrogen and nitrite were measured weekly using a DR 2000 spectrophotometer (Hach Co., Loveland, CO, USA).

2.2. Sample collection and RNA extraction

At the end of the feeding trial, for miRNA sequencing, a total of 8 crayfish were selected (female: male = 1:1) per cage. Crayfish hepatopancreas tissues were removed and immediately frozen in liquid nitrogen, and then stored at −80 °C until used for subsequent analysis. RNA was individually extracted with *mirVanaTM* microRNA Isolation Kit (Ambion) following the manufacturer's instructions. Quantities of the two groups RNAs were determined using a Nanodrop spectrophotometer (Nanodrop Technologies, USA). The RNA integrity number (RIN) was determined in an Agilent BioAnalizer (Agilent Technologies, USA). RNAs with an RIN >8.0 were further processed for the sequencing run. Equivalent RNA concentrations of 12 samples from each group were pooled for sequencing, respectively.

2.3. Small RNAs library preparation and sequencing

The small RNAs libraries were deep sequenced by Illumina Hiseq2000 according to the manufacturer's instructions (Illumina, inc., USA), using total RNA as start material. Small RNAs were first isolated from the total RNA and a pair of Illumina proprietary adaptors were ligated to their 5′ and 3′ ends. Then adaptor-ligated small RNAs were reverse transcribed to create cDNA constructs. The two generated small cDNA libraries were amplified through PCR. Subsequently, library was sequenced (BGI, Shenzhen, China).

2.4. Data cleaning, length distribution and sequence data analysis

An initial filtering step was performed to remove low quality reads, including reads with 5′ primer contaminants, reads without 3′ primer, reads without the insert tag, reads with poly A, reads shorter than 18 nt. Then the length distribution of the clean reads was summarized. The small RNA tags were mapped to *Daphnia pulex* genome (no whole genome data for *P. clarkii*) (NCBI Assembly GCA_000187875.1) by SOAP to analyze their expression and distribution on genome. We aligned the selected small RNA sequences to the genome sequence of *D. pulex*, which was the closest evolutionarily related species with an available sequenced genome, to perform a distribution analysis on a genomic scale. Non-coding RNA such as rRNA, tRNA, snRNA, etc were identified by alignment to RFam 10.1 and NCBI database. After being classified into different categories based on the sequence similarity, the remnant reads