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Hemolymph transcriptome analysis of Chinese mitten crab (*Eriocheir sinensis*) with intact, left cheliped autotomy and bilateral eyestalk ablation

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ABSTRACT

In the pond culture of *Eriocheir sinensis*, high limb-autotomy seriously affects the quality and culture's economic efficiency. Based on our previous studies, limb autotomy can induce the changes of hematological immune response in *E. sinensis* hemolymph. Eyestalk ablation can accelerate the regeneration of limbs after autotomy. To detect the important functional genes related to the hematological molecular immunity of *E. sinensis*, we compared and analyzed the hemolymph transcriptome data of the intact crab, left cheliped autotomized crabs and bilateral eyestalk ablation crabs with high-throughput sequencing techniques. The results showed that the three groups obtained 62 172 414, 68 143 682, and 67 811 618 clean reads, respectively. A total of 9567 differentially expressed genes were obtained by multiple comparison of the three groups' libraries. Gene ontology (GO) functional classification analysis shows that the differential genes belong to 42 categories of biological process, cellular components and molecular function. The differentially expressed genes in the three libraries were enriched to 344 specific KEGG metabolic pathways by KEGG enrichment analysis, such as the up-regulated gene (dual oxidase (*Duox*), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (*YWHAQ*)) in MAPK signaling pathway, the up-regulated gene (aldehyde dehydrogenase 1 (*ALDH 1*)) and down-regulated gene (UDP-glucuronosyltransferase 2 (*UGT 2*)) in metabolism of the xenobiotics by cytochrome P450 pathway, the down-regulated gene (actin gene (*AG*), heat shock protein 90 (*HSP 90*)) in fluid shear stress and atherosclerosis pathway. To verify the expression levels of DEGs identified by RNA-Seq, the above six hematological immune-related genes were selected for qRT-PCR validation, the qRT-PCR results were consistent with the DEGs results. Our research obtained abundant *E. sinensis* hemolymph transcriptome information by RNA-Seq, which provides multi-level information for the cloning of novel genes and the study of hemolymph molecular immunology mechanisms of *E. sinensis*.

1. Introduction

The Chinese mitten crab (*Eriocheir sinensis*) is an important special aquaculture species in China, which is delicious, nutritious, has high economic value, and is widely distributed in China's north-south coastal lakes and rivers [1]. At present, most of *E. sinensis*' culture models are pond mixed culture and high-density culture, which frequently lead to limb autotomy. During the pond culture of *E. sinensis*, various factors can cause a high rate of limb autotomy, such as fighting, defense and foraging, unsuccessful or unsynchronized molting and artificial harvesting [2–5]. The limb-autotomy rate of *E. sinensis* has seriously affected the survival rate, quality and economic benefits of aquaculture.

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In recent years, the effect of limb-autotomy on *E. sinensis* has mostly remained at the macroscopic level, such as feeding efficiency [6], molting cycle [7] and survival rate [8]. Although, the changes in hemolymph physiology after limb autotomy have been studied, there is still no report on the effect of limb autotomy on *E. sinensis* at the genetic level, such as molecular immunology [9]. In addition, as an important neuroendocrine regulatory organ of crustaceans, the eyestalk plays an important role in molting, growth and gonadal development [10]. At present, it is very common for shrimps and crabs to ablate the eyestalk to promote molting, further accelerating the regeneration of the limb [1,11]. However, limb autotomy or eyestalk ablation will bring negative impacts on the immunity and antibacterial response of crustaceans [9,12–14]. However, the molecular mechanism of their immune regulation has not been reported.

Unlike vertebrates, the crustacean's immune system is composed of the innate immune system, which includes hematological and cellular immunity. Hematological immunity mainly involves various hematological immune factors and immune-related enzymes in the hemolymph, such as heat shock protein 90 (HSP 90), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein, YWHAQ), dual oxidase (Duox), aldehyde dehydrogenase 1 (ALDH 1), Cathepsin L (CatL) and UDP-glucuronosyltransferase 2 (UGT 2) [15–19]. Hemocyte immunity mainly includes phagocytosis, package action, agglutination and melanization of hemocyte [20]. These immune regulators will function through one or some metabolic pathways. The MAPK signaling pathway is an important signaling system that mediates cellular response, which participates in the processes of cell growth, development, division, death, immune defense and stress response [21], *Duox* and *YWHAQ* belong to this pathway. Cytochrome P450 is involved in a variety of metabolic and biosynthetic processes, of which the cytochrome P450 enzyme is a multifunctional enzyme that is involved both in the biotransformation of exogenous substances and in the metabolism of endogenous substances [22], *ALDH 1* and *UGT 2* belong to this pathway. The fluid shear stress and atherosclerosis pathway include many immune-related proteins (e.g. HSP 90, AG, hioredoxin 1 (Trx 1)) and related enzymes (cathepsin L (Cts L), which are associated with body immunity, substance metabolism, cell structure and function maintenance, showed a significant down-regulation [23,24].

In addition, when the crustacean suffers from an external injury, the hemolymph and hemocyte are the quickest and most effective ways to exert immunity and wound repair. So far, there have been no reports on the hemolymph molecular immunological mechanism of *E. sinensis* after limb autotomy and eyestalk ablation. The development of high-throughput sequencing technology has become an important tool for studying the functional genomics of many species, especially exploring the molecular mechanisms of some biological phenomena. In this study, we performed hemolymph transcriptome comparisons between intact crabs, left cheliped autotomized crabs and bilateral eyestalk ablation crabs using transcriptomic high-throughput sequencing technology and bioinformatics methods. Our study will provide new insights into the hematological immunity and biological responses of the species to limb autotomy and eyestalk ablation.

2. Materials and methods

2.1. Experimental crabs and ethics

All experimental protocols were reviewed and approved by the Animal Bioethics Committee, Shanghai Ocean University, China. Sampling operations complied with the IACUS Guidelines for the Care and Use of Animals in Scientific Experiments. During early July 2017, 30 male healthy and intact *E. sinensis* (Crustacea; Decapoda; and Grapsidae) (13.25 g ± 2.92 g) were collected from the earth pond at the Chongming research base of Shanghai Ocean University (Shanghai, China). Crabs were randomly divided into three groups (10 crabs each group): 1) control group (C); 2) left cheliped autotomized (LCAu), where autonomy was achieved by gently grasping the left cheliped using the researcher's fingers and then the crab would spontaneously autotomize the corresponding limb; and 3) bilateral eyestalk ablation group (BESA), where eyestalk ablation was performed by clipping the eyestalk using sterile scissors and immediately cauterizing the wound to prevent the loss of hemolymph and avoid infection. Before the start of the experiment, all crabs used for the experiment were anesthetized on ice, and both the crabs and all the experimental tools were wiped with sterile 75% alcohol cotton balls.

Table 1
Primer information for quantitative real-time polymerase chain reaction.

Primers	Sequences (5′-3′)
Duox-F	TTGGCTTCTGGTCTGAGGAG
Duox-R	CATGTGTCAACACAGCCAGT
YWHAQ-F	GGAATGGAGATGTGTAAATAGG
YWHAQ-R	ATCAGACAGTGCAGGAGAAGA
ALDH1-F	CATCCGCAACCTGGAGGTCA
ALDH1-R	CTCCCGAGTTGAAGGGTTACAT
UGT2-F	AAAAACAGGAATGCCCAGGAC
UGT2-R	AAGTGGCTACCCCAGCAAGAC
AG-F	GCTGTTGTGACAAAAGAATAAC
AG-R	AGTGCCCATCTATGAAGGTTA
HSP90-F	GAAGGTGATCCGCAAGAACC
HSP90-R	GTTGGTGGAGTCCTCATGGA
β-actin -F	TCATCACCATCGGCAATGA
β-actin -R	TTGTAAGTGGTCTCGTGGATG

Table 2
Evaluation of RNA-Seq Data of *E. sinensis* with different treatment.

	C	BESA	LCAu
Raw reads	65 201 612	71 413 202	70 693 958
Clean reads	62 172 414	68 143 682	67 811 618
Clean ratio (%)	95.35	95.42	95.92
rRNA trimed	61 819 301	67 878 007	67 536 543
rRNA ratio (%)	0.57	0.39	0.41

Note: Clean ratio = (Clean reads/Raw reads) * 100%; rRNA ratio = [(Clean reads – rRNA trimed)/Clean reads] * 100%.

2.2. Sample collection

After treatment, hemolymph was drawn using a sterile 1-mL syringe from the unsclerotized membrane of the right third pereopods and was immediately diluted 1:1 with sterile anticoagulant (30 mM trisodium citrate, 338 mM NaCl, 115 mM glucose, and 10 mM EDTA). The mixture was centrifuged at 12000 r/min for 10 min, after which the supernatant was discarded, and the precipitate was collected and stored in liquid nitrogen for RNA isolation.

2.3. RNA isolation and RNA-Seq library preparation

In each group of 10 samples, two samples were randomly selected and mixed for 5 biological replicates in each group. Total RNA was extracted from collected hemolymph using RNAiso™ plus reagent (RNA Extraction Kit, TaKaRa, Japan) according to the manufacturer's protocol. The total RNA concentration, integrity and quality were estimated using a micro-volume ultraviolet–visible spectrophotometer (Quawell Q5000; Thmorgan, China) and agarose-gel electrophoresis, respectively. The RNA integrity number (RIN) of all the RNA samples above 8.0 was used for RNA-Seq library construction using the Illumina Truseq™ RNA sample Prep Kit (Illumina, USA). Then, we used the Illumina Truseq™ 2000 sequencing platform to complete the transcriptome sequencing. The transcriptome sequencing of the target