Genome-wide identification of Chinese shrimp (*Fenneropenaeus chinensis*) microRNA responsive to low pH stress by deep sequencing

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Abstract

pH has a great impact on the distribution, growth, behavior, and physiology in many aquatic animals. Here, we analyzed miRNA expression profiles of Chinese shrimp (*Fenneropenaeus chinensis*) from control pH (8.2) and low pH (6.5)-treated shrimp. Expression analysis identified 6 known miRNAs and 23 novel miRNAs with significantly different expression between control pH 8.2 and low pH 6.5; the predicted target genes of differentially expressed miRNAs were significantly enriched in organic acid metabolic process, oxidoreductase activity, coenzyme binding, cofactor binding, and collagen trimer. Moreover, target genes were significantly enriched in several Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways including citrate cycle, pyruvate metabolism, cytokine-cytokine receptor interaction, tight junction, carbon metabolism, etc. Our survey expanded the number of known shrimp miRNAs and provided comprehensive information about miRNA response to low pH stress.

Keywords *Fenneropenaeus chinensis* · microRNA · Low pH stress · HiSeq sequencing

Introduction

Because of its rich taste and large size at harvest, Chinese shrimp, *Fenneropenaeus chinensis*, has become one of the main cultured aquatic animals, especially in northern China. The pH range 7.0–8.5 is known to be optimum for aquatic animals. In the natural water bodies or the culture ponds where *F. chinensis* and other

crustaceans occur and are cultured, pH levels often fluctuate. For example, the pH levels in water bodies decreased when ponds were built in the areas with acid sulfate soils or areas with acid precipitation (Haines 1981). Furthermore, excessive freshwater or storm rain might rapidly change the pH levels in a short period of time, whilst the sludge, made up of shrimp feces and feed residues, cause pH levels to change in the long term during cultivation (Lemonnier et al. 2004; Zhou et al. 2009). In general, crustaceans have the ability to adapt to a variety of pH levels, as well as other environmental perturbations. It is also evident that abnormal pH especially lower pH triggers many changes and responses in physiological, biochemical, and molecular pathways thus ultimately having an impact on various cellular and whole organism processes. Adverse reactions including retarded growth, reduced rates of cyst hatching, ingestion, respiration and feeding, increased ammonia excretion rates, decreased immune parameters, disturbed ion regulation, and acid-base imbalance (Allan and Maguire 1992; Chen and Lee 1997; Chen 2003; Chen et al. 2015; Haddaway et al. 2013; Kawamura et al. 2015; Kim et al. 2015; Morgan and McMahon 1982; Zheng et al. 2015) are reported in many crustaceans.

However, crustaceans have evolved excellent defense mechanisms to protect themselves from abnormal pH conditions. For example, when confronting alkaline pH stress, *Cherax quadricarinatus* mounts a robust transcriptional

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response affecting several genes, such as cytoplasmic carbonic anhydrase (CA), Na^+/K^+ -ATPase (NKA), and vacuolar-type H^+ -ATPase (HAT) which have been shown to play important roles in the maintenance of alkaline-acid equilibrium (Ali et al. 2017; Liu et al. 2015b). Brine shrimp generate more antioxidant enzymes like peroxidase to reduce the intracellular toxicity and protect the cell membrane in response to acidification stress resulting in the generation of reactive oxygen species (Zheng et al. 2015). Crayfish with calcified exoskeletons have compensated for impaired calcium absorption and a resultant reduction in layer thickness by depositing more lamellae under low pH stress (Haddaway et al. 2013).

In recent years, however, a class of short endogenous non-coding RNAs termed miRNAs was shown to regulate various biological processes, especially by mRNA degradation or suppression of the translation process at the post-transcriptional level, whether under biotic or abiotic stress conditions (Lucas and Raikhel 2013). In tilapia, the cross talk between vascular endothelial growth factor (VEGF) and Ang-2 in hypoxia-induced microvascular dysfunction under hypoxia conditions was directly regulated by *miR-204* (Zhao et al. 2014a, b). Zhang et al. reported that a high-fat diet might result in the hepatic steatosis influenced by miRNA expression in the liver of *Megalobrama amblycephala* (Zhang et al. 2014). In crustaceans, 12 miRNAs exhibited differential expression patterns in *Portunus trituberculatus* after low salinity stress. *miR-2788* proved to have an important role in hyper-osmoregulation by regulating the expression of S-adenosylmethionine synthetase (SAM) (Lv et al. 2016). In shrimp *Penaeus monodon*, *pmo-miR-315* and *pmo-miR-750* were identified as highly responsive miRNAs against white spot syndrome virus infection (WSSV), suggesting that miRNAs might have an important role in regulating innate immune response.

Up to now, few studies have been conducted on how miRNAs are involved in the response of crustaceans to low pH stress. Consequently, the aim of this study was to determine the identity and abundance of low pH-responsive miRNAs in *F. chinensis*. We constructed two RNA libraries (control pH 8.2 and low pH 6.5) and sequenced with an Illumina HiSeq2000 sequencing platform. The present study provides the first suggestion of the potential roles of miRNAs and associated mechanisms in the shrimp's response towards low pH stress.

Materials and methods

Animals, materials, and low pH stress treatment

Healthy Chinese shrimp (wet weights, 17.25–19.35 g), “Huanghai No. 3,” were originated from “Huanghai No. 1,” a variety that was cultivated widely in northern China and were obtained from a shrimp farm in Changyi, China. Animals were acclimated in tanks with pH 8.2 ± 0.21 for

3 weeks before the experiment. Thirty shrimps were distributed into two groups in separate tanks. One group with pH 8.2 was used as the control, and the other was treated with low pH 6.5. Low pH was adjusted with gradual mixing with HCl (1 M). Hepatopancreas were extracted from three individuals as biological replicates and immediately frozen in liquid nitrogen and stored at -80°C for RNA extraction.

sRNA library construction and sequencing

Total RNAs were extracted using TRIzol reagent (Invitrogen Corp., Carlsbad, CA) and treated with RNase-free DNase I (Promega Corp.) to remove genomic DNA contamination. Two separate sRNA libraries were generated from control and low pH-treated shrimp hepatopancreas and subjected to sequencing using an Illumina HiSeq2000 sequencing platform.

Identification of low pH-responsive miRNAs and functional analysis of target genes

Homology-based searching against the public database, miRbase (<http://www.mirbase.org/>), was used to annotate conserved miRNAs, and miRdeep (<http://www.australianprostatecentre.org/research/software/mirdeep-star>) was used to predict novel miRNAs. Differentially expressed miRNAs were screened using DEGSeq analysis tool (<http://bioinfo.au.tsinghua.edu.cn/software/degseq>) (Wang et al. 2010). Only the miRNAs that showed $\log_2$ fold change ≥ 1 and FDR cutoff < 0.05 were determined to be miRNAs that were differentially expressed between low pH and control groups. The miRNA target gene database, RNAhybrid (<https://bibiserv.cebitec.uni-bielefeld.de/rnahybrid/>), and miRanda (<http://www.microrna.org/microrna/home.do>) were used to predict target genes of the differentially expressed miRNAs.

Gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis

WEGO software (<http://wego2.genomics.org.cn>) was used to obtain gene ontology (GO) terms of the target genes of miRNAs. KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway analysis was carried out to classify the enriched functional groups. The significance of GO term enrichment and the pathway analysis was defined by *p* value and FDR (a *p* value < 0.05 is recommended).

Validation of miRNA expression using qRT-PCR

Five conserved and eight novel miRNAs were randomly selected and investigated the expression profile on StepOne™ Real-Time PCR system (Applied Biosystems, USA). The total RNA used in the assay was extracted from the hepatopancreas of

Fig. 1 Small RNA library sequencing and annotation. **a** Length distribution of small RNA reads. **b** Annotation of small RNAs. The percentages of each type of sRNAs are indicated in brackets

F. chinensis with TRIzol reagent (Invitrogen). The polyadenylation and reverse transcription was carried out in a 10 μ L volume containing 2 μ g total RNA, 5 μ L mRQ Buffer (2 $\times$) (Takara), and 1.25 μ L mRQ Enzyme Mix (Takara). The reaction was performed as follows: 37 $^{\circ}$ C for 60 min and 85 $^{\circ}$ C for 5 min. Before the real-time RT-PCR assay, 90 μ L ddH₂O was added to bring the total volume of cDNA sample to 100 μ L. The real-time RT-PCR was carried out in a 15 μ L reaction mixture containing 4.6 μ L ddH₂O, 7.5 μ L SYBR Premix Ex TaqTM, 0.3 μ L ROX Dye (50 $\times$) (Takara), 0.2 μ L of each primer (10 μ M), and 2.0 μ L of the prepared cDNA sample. The data were analyzed using the $2^{-\Delta\Delta C_t}$ method. The expression of miRNAs was normalized against U6 snRNA levels. All the primers used in this study are listed in Online Resource 1.

Results

Computational identification of miRNA from sRNA libraries

To identify low pH-responsive miRNAs in *F. chinensis*, two sRNA libraries from the control and low pH-treated shrimp hepatopancreas were constructed and sequenced on an Illumina HiSeq2000 to yield 17,185,135 and 17,987,351 raw reads, respectively. After removing low-quality sequences shorter than 16 nucleotides, short reads, contaminants, and adapter-null reads, 14,875,579 (86.56%) and 15,863,698 (88.19%) clean reads were obtained, respectively. Within these sRNAs, 311,625 and 529,969 reads were classified as

miRNA sequences, accounting for 1.88 and 3.59% of all the sRNA reads, respectively. The length distribution of miRNAs identified from the two libraries showed a similar pattern with a predominant size of 22 nt, following by 21- and 23-nt classes (Fig. 1). A total of 204 miRNAs were detected in *F. chinensis*, of which 11 (5.4%) were conserved and 193 (94.6%) were novel (Online Resource 2).

Identification of differentially expressed miRNAs

Differential expression analysis of the two libraries was conducted with DESeq R package (1.10.1), and miRNAs with significant differential expression (p value < 0.05, log₂ fold change > 1) were considered as truly differentially expressed between control and low pH groups. Of all the 204 miRNAs, 29 were labeled as differentially expressed miRNAs, of which 13 (0 conserved and 13 novel) miRNAs were upregulated and 16 (6 conserved and 10 novel) were downregulated. In the present work, let-7e and let-7c were the two conserved miRNAs that showed the highest downregulation (4.50-fold) and upregulation (3.98-fold), respectively (Table 1). Another ten miRNAs were detected as exclusively expressed (Readnum > 10) in pH 6.5 and control groups (Online Resource 3).

GO and KEGG pathway analyses

GO analysis of these target genes was performed to gain further insight to the miRNA functions by using RNAhybrid and miRanda. These putative targets of the differentially expressed miRNAs were clustered into three GO

Table 1 The differentially expressed miRNAs

miRNA	Control reads	pH 6.5 reads	Log2 ratio (pH 6.5/control)	Regulated	Sequence
let-7e	362	16	-4.50	Down	ugagguaguagauugaaau
novel_mir155	18	1	-4.17	Down	acgaggaccuugagcugaaca
let-7d	586	34	-4.11	Down	cuauacgaccugcugccuuucuuag
novel_mir187	16	1	-4.00	Down	gugaaaucaaccgguccucaa
novel_mir108	15	1	-3.91	Down	cugauucgucugugugagaguc
novel_mir69	13	1	-3.70	Down	ugggaauaggaaguuaggagagg
novel_mir41	33	3	-3.46	Down	ugacugccgugugcugggccuugc
novel_mir32	19	2	-3.25	Down	cggacugcacggcguggaccccg
novel_mir72	119	18	-2.72	Down	caggggcuauaucugauagagg
novel_mir62	31	5	-2.63	Down	ugggcuagaacugaggcaaggacu
let-7i	690	121	-2.51	Down	ugagguaguaguuugugcu
novel_mir66	171	34	-2.33	Down	ucucugagcuucugcaguguguc
novel_mir118	102	23	-2.15	Down	augggcuucgcgggagagagcgg
let-7c	133	32	-2.06	Down	ugagguaguaguuugauguuu
let-7	434,112	155,557	-1.48	Down	ugagguaguaguuugauagua
bantam-a	92	37	-1.31	Down	ugagaucacuaugaaagcugg
novel_mir102	1	19	4.25	Up	uggguauugugaguuugaacag
novel_mir28	1	12	3.58	Up	gggggcgcgcggauguaa
novel_mir225	1	11	3.46	Up	acgguuuuggcugagugacgac
novel_mir7	2	20	3.32	Up	aaucgaucuaacggcaacacauuu
novel_mir116	6	56	3.22	Up	cgaagaauacaguagaccuu
novel_mir215	2	14	2.81	Up	uggaauuguaugcugcagugagc
novel_mir159	6	29	2.27	Up	uagguucugaaagauucugauggg
novel_mir260	4	16	2.00	Up	cagguuuuagagcaguuugagagu
novel_mir9	10	37	1.89	Up	ucuguuauucugcucugcucu
novel_mir3	9	30	1.74	Up	ugcuuuccgucgcauuugacucguu
novel_mir39	1717	5031	1.55	Up	uccggagcagaagucuaacu
novel_mir146	429	1158	1.43	Up	gauggaauacgggagaauugcgau
novel_mir151	58	117	1.01	Up	ugugugugugugugugug

terms including biological processes, molecular functions, and cellular components. According to the numbers of predicted target genes, cellular process, metabolic process, and single-organism process were recognized as the top GO biological process terms; catalytic activity and binding were top terms in molecular functions (Online Resource 4). GO enrichment analysis of target genes produced significantly enriched terms ($p < 0.05$), including organic acid metabolic process, oxidoreductase activity, coenzyme binding, collagen trimer, ligase activity, nucleobase catabolic process, and carboxylic acid metabolic process (Fig. 2).

To investigate the pathways and molecular interactions of the identified targets genes, we carried out a KEGG analysis. The results showed that the target genes of differentially expressed miRNA were classified into six different categories: cellular processes, environmental information processing,

genetic information processing, human diseases, metabolism, and organismal systems (Online Resource 5). Of these, ten functional pathways appeared to be significantly enriched after exposure to low pH ($p < 0.05$). As shown in Fig. 3, the enriched pathways were primarily focused on carbohydrate metabolism (TCA cycle, pyruvate metabolism, and glyoxylate and dicarboxylate metabolism), signaling molecules and interaction (cytokine-cytokine receptor interaction and hippo signaling pathway), and excretory system (proximal tubule bicarbonate reclamation).

Verification of miRNA expression by qRT-PCR

Expression of differentially expressed miRNAs was further validated by qRT-PCR. The differential expression profiles of 13 randomly selected miRNAs (8 novel and 5 known)

Fig. 2 GO enrichment analysis of predicted target genes for differentially expressed miRNAs

Fig. 3 Pathway enrichment analysis of predicted target genes for differentially expressed miRNAs

were verified by qRT-PCR. As revealed by high-throughput sequencing results, qRT-PCR exhibited a similar expression trend, where six miRNAs (novel_mir118, novel_mir155, let-7c, let-7d, let-7e, let-7i) were downregulated and six (novel_mir9, novel_mir3, novel_mir28, novel_mir215, novel_mir159, novel_mir102) were upregulated. However, the expression pattern of bantam-a detected in qRT-PCR was inconsistent with miRNA-Seq results. Regression analysis of the two approaches indicated a strong correlation with $R^2 = 0.820$ (Fig. 4). This was likely due to sequence bias introduced by small RNA libraries or profiling miRNA qRT-PCR or to different normalization approaches employed in these two strategies.

Discussion

The pH is an important environmental factor affecting the life of aquatic animals that can become stressed or die when exposed to pH extremes. In recent years, miRNAs have received considerable attention with a number of authors reporting their important roles in response to abiotic stresses, including heat (Liu et al. 2015a), salinity (Lv et al. 2016; Sun et al. 2016), hypoxia (Sun et al. 2016), anoxia (Biggar and Storey 2012), and nutrient restriction (Zhu et al. 2015). The role of miRNAs in response to abnormal pH, especially low pH, is poorly understood. To provide additional insights on the molecular mechanisms of *F. chinensis* against low pH stress, we

Fig. 4 Selected miRNAs were experimentally tested by qRT-PCR confirming expression patterns of deep sequencing. **a** Most miRNAs were consistent with the expression patterns of deep sequencing. **b** Regression analysis between sequencing and qRT-PCR

determined low pH-responsive miRNAs through an Illumina HiSeq 2000 sequencing technique and elucidated the biological functions of target genes. A total of 29 miRNAs was significantly differentially expressed following exposure to low pH condition. Among these, 13 upregulated miRNAs and 16 downregulated miRNAs were determined, and corresponding targets were predicted (Table 1). Nine miRNAs were exclusively expressed in pH 6.5, and one was exclusively expressed in control groups, respectively (Online Resource 6).

Identification of putative miRNA targets and enrichment analysis in biological processes and metabolic pathways are two effective strategies to provide evidence of molecular mechanisms modulated by miRNAs when exposed to low pH stress. From functional annotation and enrichment analysis, we can infer that a variety of biological processes modulated by miRNAs, including acid metabolism (organic acid metabolic process, carboxylic acid metabolic process) and catalytic activity (oxidoreductase activity, coenzyme binding, ligase activity, nucleobase catabolic process), were activated to act as important molecular mechanisms for *F. chinensis* against low pH stress (Fig. 1). Organic acids, usually as the end-products from glucose fermentation, are mainly produced in mitochondria through the tricarboxylic acid cycle pathway and reflect the metabolic activity of the cell (López-Bucio et al. 2000). In *Bacillus cereus*, the production of three major organic acids, lactate, formate, and acetate, was lower at pH 5.5 compared with pH 7.0. Proton consumption and excretion caused by many enzymatic reactions were regarded as important protection mechanisms in intracellular pH homeostasis (Le Lay et al. 2015). Similar results were also seen in *F. chinensis*, indicating that these processes provide an adaptive response against acid environments under low pH conditions.

Ten pathways were recorded as significantly enriched for low pH adaptation, in which citric acid (TCA) cycle and pyruvate metabolism were the top two enriched pathways (Fig. 3, Online Resource 7, 8). TCA is the most important metabolic pathway for energy supply in animal tissues. There is evidence that TCA cycle intermediates succinate and α -ketoglutarate (α KG), the ligands for Sucnr1 and OXGR1, may play a vital role in a variety of stress responses. OXGR1 can detect the α KG and regulate Cl^- -dependent HCO_3^- secretion and NaCl reabsorption and is essential for the reabsorption of salt during pH disturbances (Peti-Peterdi 2013). Another significantly enriched pathway of the differentially expressed miRNAs is pyruvate metabolism (Fig. 2). When the pH homeostasis is in disorder, the acid-base balance is maintained via the proton-extruding and proton-importing processes which were motivated by transmembrane potential. As ATP stores, the energy for proton extrusion, are depleted, pyruvates accumulate due to anaerobic metabolism of glycogen (Casey et al. 2010). The toxic levels of intracellular pyruvate, in turn, can be reduced at low pH, and this may be achieved by the conversion of pyruvate into C4 compounds and their subsequent excretion (Repizo et al. 2011). Enhanced

pyruvate metabolism was observed in this study (Online Resource 4), indicating that high energy demand for proton extrusion might be required for *F. chinensis* to prevent gradual accumulation of protons in response to low pH, promoting the generation of pyruvates. Tight junctions have a paracellular barrier function to control the passage of ions and large solutes. In addition, tight junctions participate in the recruitment of cytoskeletal and signaling molecules at their cytoplasmic surfaces (Terry et al. 2010). Hsu verified that under low pH, when chitosan is positively charged, the electrostatic interaction between chitosan and integrin $\alpha_v\beta_3$ leads to the conformational change of integrin and its clustering along the cell border, thus enhancing the paracellular permeability and disrupted tight junction (Hsu et al. 2013). In *F. chinensis*, the expression of many genes involved in tight junction pathways was changed (Online Resource 9), indicating that tight junction functions might be interrupted upon exposure to low pH (6.5).

To characterize the mechanisms underlying acid stress response of *F. chinensis*, we conducted this study using a HiSeq2000 sequencing platform to screen and characterize low pH stress-associated miRNAs from two small RNA libraries isolated from control and low pH-stressed shrimp hepatopancreas. The expression profile and subsequent GO and pathway analysis provided additional insights on molecular mechanisms that *F. chinensis* developed to undergo the stress response induced by low pH conditions.

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