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Potential role for microRNA in facilitating physiological adaptation to hypoxia in the Pacific whiteleg shrimp *Litopenaeus vannamei*

Wei Wang, Ping Zhong, Jun-Qiao Yi, Ai-Xuan Xu, Wen-Yi Lin, Zhen-Cong Guo, Cheng-Gui Wang, Cheng-Bo Sun**, Siuming Chan*

College of Fisheries, Guangdong Ocean University, Zhanjiang, Guangdong, PR China

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ABSTRACT

Hypoxia is one of the most common physiological stressors in shrimp farming. Post-transcriptional regulation by microRNAs has been recognized as a ubiquitous strategy to enable transient phenotypic plasticity and adaptation to stressful environment, but involvement of microRNAs in hypoxia stress response of penaeid shrimp remains elusive. In this study, small RNA sequencing and comparative transcriptomic analysis was conducted to construct a comprehensive microRNA dataset for the whiteleg shrimp *Litopenaeus vannamei* exposed to hypoxia challenge. A total of 3324 known miRNAs and 8 putative novel miRNAs were identified, providing a valuable resource for future investigation on the functional mechanism of miRNAs in shrimp. Upon hypoxia, 1213 miRNAs showed significant differential expression, and many well-known miRNAs involved in hypoxia tolerance such as miR-210, let-7, miR-143 and miR-101 were identified. Remarkably, the vast majority of these miRNAs were up-regulated, suggesting that up-regulation of miRNAs may represent an effective strategy to inhibit protein translation under stressful hypoxic condition. The differentially expressed miRNAs were potentially targeting a wide variety of genes, including those with essential roles in hypoxia tolerance such as *HIF1a* and *p53*. GO and KEGG enrichment analysis further revealed that a broad range of biological processes and metabolic pathways were over-represented. Several GO terms associated with gene transcription and translation and KEGG pathways related to cytoskeleton remodeling, immune defense and signaling transduction were enriched, highlighting the crucial roles of these cellular events in the adaptation to hypoxia. Taken together, our study revealed that the differentially expressed miRNAs may regulate host response to hypoxia by modulating the expression of stress response genes such as *HIF1a* and *p53* and affecting key cellular events involved in hypoxia adaptation. The findings would expand our knowledge of the biochemical and molecular underpinnings of hypoxia response strategies used by penaeid shrimp, and contribute to a better understanding of the molecular mechanisms of hypoxia tolerance in decapod crustaceans.

1. Introduction

Hypoxia, or oxygen deficiency, is one of the most common physiological stressors in aquatic environments [1,2]. In aquaculture ponds, due to a variety of factors such as high cultivation density, extensive use of commercial feeds and deterioration of water quality, dissolved oxygen level in water fluctuates frequently and can drop below animal's physiological limit [3,4], posing significant threats to the cultured animals [5]. Like other aquatic animals, shrimp are very susceptible to hypoxia [6]. Low concentrations of dissolved oxygen can cause damage to the gill, hepatopancreas and hemolymph of shrimp and compromise the immune system, leading to outbreaks of disease and death of the animals [7]. Consequently, a considerable amount of research effort has been devoted to elucidate the effects of hypoxia on the growth, reproduction and immunity of shrimp [4,6,8].

The Pacific whiteleg shrimp, *Litopenaeus vannamei*, is an important crustacean species for aquaculture throughout the world. Its annual production constitutes about 70% of the total yield of shrimp aquaculture in the world [6]. Since its introduction to China in the 1980s, *L. vannamei* has quickly became the dominant shrimp species for artificial cultivation thanks to its superior characteristics such as high growth rate, strong stress tolerance and euryhalinity [9]. However, with the deterioration of water quality and lack of good shrimp strains that are selectively bred for local culturing environment, the production of *L.*

* Corresponding author.
** Corresponding author.
E-mail addresses: scb248@126.com (C.-B. Sun), siuming573@sina.cn (S. Chan).

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vannamei in China has been declining in recent years [9], and hypoxia has become one of the main abiotic factors that constrain the survival rate of cultured shrimp [4]. Hence, a deeper understanding of the physiological responses to hypoxia in *L. vannamei* is not only of great importance for the sustainable development of shrimp aquaculture industry [10], but offers more insights into the molecular mechanisms of hypoxia tolerance in crustaceans as a whole [11].

MicroRNAs (miRNAs) are a group of non-coding RNAs approximately 22 nucleotides in length [12]. By means of base pair matching to specific regions of target mRNAs, miRNAs can lead to degradation or translational stagnation of target mRNAs [13]. With more than 60% of mammalian mRNAs predicted as their target, miRNAs have been reported to be implicated in a variety of fundamental cellular processes including apoptosis, cell differentiation, autophagy, signal transduction, and immune defense [13–15]. Recently, a growing body of evidence from some classic model organisms has supported that miRNAs also play essential roles in the adaptation of living organisms to stressful environments [13,16,17]. For example, upregulation of mir-34 in *C. elegans* is necessary for developmental arrest and adaptation to a lower metabolic state to protect animals against stress-related damage [18], miR-7 knockout flies develop abnormal eyes when exposed to alternating temperatures [19], and zebrafish deficient in miR-8 aren't able to respond to osmotic stress [20].

Although studies on the role of miRNAs in mediating stress responses are gaining momentum in several well-established model organisms [21], similar research on this topic in the large community of aquatic invertebrates including shrimp is still in its infancy [11,22]. However, the advancement of high-throughput sequencing technologies such as small RNA sequencing has provided unprecedented opportunities to explore this question in aquatic invertebrates [23,24]. Some recent studies using this approach on aquatic animals have revealed that miRNAs are also involved in a wide range of stress responses in these lower taxa, such as heat stress response of the coral *Acropora digitifera* [22], adaptation against desiccation in the oyster *Crassostrea gigas* [25], response to low salinity stress in the swimming crab *Portunus trituberculatus* [26], and physiological tolerance of hypoxia in the oriental river prawn *Macrobrachium nipponense* [11]. Although it has been speculated that miRNAs are also likely to participate in the regulation of hypoxia stress response in peneaid shrimp [23], empirical evidence from these taxa supporting this hypothesis is still scarce.

To further examine hypoxia stress response in the whiteleg shrimp *L. vannamei* and explore the functions of miRNAs in hypoxia tolerance in penaeid shrimp, small RNA sequencing and comparative transcriptomic analysis was conducted in this study to construct a comprehensive microRNA dataset for *L. vannamei*. Two small RNA libraries from gill tissues of *L. vannamei* under normal condition or exposed to hypoxia challenge were sequenced and compared, and differentially expressed miRNAs (DEMs) as well as their potential target genes were analyzed. The data obtained could help to illuminate the role of miRNAs in regulating hypoxia stress response in shrimp, and may contribute to the development of effective strategies for minimizing hypoxia caused damage in shrimp farming.

2. Material & method

2.1. Sample collection

Shrimp used in the experiment were obtained from the Donghai Island Aquaculture Center of Guangdong Ocean University. To minimize the effect of different body sizes, only shrimp with a body length of ~6 cm and a body weight ~2 g were collected. Prior to experiment, the animals were acclimated for 2 weeks in cycling plastic tanks with aerated seawater (25 ± 2 °C; salinity 35‰). During the acclimation period, shrimp were fed twice daily with commercial shrimp diet. As a common practice, feeding was suspended 12 h before the experiment to clear the intestine content of the shrimp. As shrimp at different molting stages may have divergent physiological response to hypoxia, only apparently healthy shrimp at the inter-molt stage were selected for the experiment. The molting stage was determined by microscope examination of the uropoda according to methods in previous studies [27].

2.2. Hypoxia treatment

A control group and a treatment group were included in the study. The control group was maintained under normoxic conditions with constant aeration through air-diffusing stones, and the dissolved oxygen (DO) level was continuously monitored throughout the experiment. The average DO was around 5.5 mg O_2 L^{-1} which was similar to another study [6]. For the hypoxia treatment group, the DO level was set at 1.6 ± 0.25 mg O_2 L^{-1} as previously described [6]. The hypoxia conditions were maintained by bubbling with N_2 gas until the desired O_2 concentration was obtained. The DO level was measured every half hour with an oximeter and adjusted if needed. The animals were exposed to hypoxia conditions for 24 h, and the experiments were conducted in triplicate tanks for both the control and treatment groups, with ten shrimp in each tank. Gill tissues from three shrimp in each replicate tank in the hypoxia treatment group were dissected. In parallel, gill tissues from three shrimp that were held in normoxic water for 24 h were collected as the control samples. All samples were flash frozen in liquid nitrogen and then stored at −80 °C for later use.

2.3. RNA extraction, library construction and small RNA sequencing

Total RNA was extracted from each sample using miRNA isolation kit (Ambion, USA) following the manufacturer's protocol. RNA quality and integrity of the samples were checked using the Agilent 2100 Bioanalyzer system (Agilent Technologies). RNA concentration was measured using the Qubit RNA Assay Kit in the Qubit 2.0 Flurometer (Life Technologies). Equal amount of RNA from each replicate sample for the two groups were mixed to generate a control and a treatment RNA pool, respectively. The sequencing libraries were further constructed using NEBNext Multiplex Small RNA Library Prep Set for Illumina (NEB) following manufacturer's instructions. Briefly, small RNAs ranging from 18 to 30 nt were separated by denaturing 15% polyacrylamide gel. Then ~20 μg RNA was ligated with proprietary 5′ and 3′ end adaptors provided by Illumina. The RNA was then subjected to first strand cDNA synthesis using Superscript II reverse transcriptase (Invitrogen), and 15 cycles of PCR amplification was further performed using small RNA primer set developed by Illumina. After purification of PCR products, DNA fragments were separated and dissolved in elution buffer. The library quality was firstly assessed on the Agilent 2100 Bioanalyzer system (Agilent Technologies). Then the two small RNA libraries were sequenced on an Illumina Hiseq 2500 platform.

2.4. Annotation of miRNA sequencing data

After sequencing, the raw sequencing reads were first subjected to quality check to remove low quality sequences (reads having > 50% bases with quality score ≤5), adapter-contaminated reads and reads with proportion of ambiguous sites (N) larger than 10%. Only the remaining reads were regarded as clean reads and used in following analysis. The clean reads were then aligned against the GenBank (http://blast.ncbi.nlm.nih.gov/) and the Rfam databases (http://www.sanger.ac.uk/software/Rfam) to identify potential rRNA, tRNA, snRNA, snoRNA, mRNA, and repeat sequences. These reads were discarded and not used in downstream analysis. As there was no reference genome available for *L.vannamei*, the remaining reads were searched against miRNAs from all animals in miRBase 21.0 (http://www.mirbase.org/) using the BLAST tool to identify known miRNAs. To predict potential novel miRNAs, the unannotated reads from last step were further

analyzed in terms of the secondary structure, dicer cleavage sites and the minimum free energy using the mirdeep2 software [28].

2.5. Differential expression of miRNAs and functional enrichment of their target genes

To detect differentially expressed miRNAs between the control and treatment groups, the expression of miRNA in the two libraries were normalized to TPM (transcripts per million) value based on methods in previous studies [23,29]. Differentially expressed miRNAs in response to hypoxia were identified by pairwise comparisons between the control and treatment samples using the DESeq R package [30]. The fold-change of expression level for each miRNA between the two samples and P-value were calculated based on the normalized expression values. A corrected P-value < 0.05 and a $|\log_2(\text{fold-change})|$ value > 1 were applied as the threshold for significantly differential expression. As the genome sequence of *L. vannamei* is not available, 5′ and 3′- UTR sequences were extracted from its transcriptome assembly [23] and used to predict potential target genes of the identified miRNAs using the miRanda [31]. For the target genes of differentially expressed miRNAs, Gene Ontology (GO) enrichment analysis was conducted using the GOseq package based on non-central hyper-geometric distribution method. In addition, The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to analyze functions and pathways associated with putative target genes of the DEMs. Only GO terms with a Bonferroni-corrected P value ≤ 0.05 and pathways with a FDR ≤ 0.05 were regarded as significantly enriched.

2.6. Validation of differential miRNA expression by real-time quantitative PCR (qPCR)

To estimate the accuracy of differential expression analysis for the miRNAs, real-time quantitative PCR (qPCR) was performed for selected miRNAs showing various magnitudes of expression changes. RNA samples from the control and treatment groups as mentioned above were used for stem-loop qPCR analysis. The miRNAs were first reverse transcribed using TaqMan microRNA Assays Kit (Applied Biosystems) as mentioned in previous studies [23]. Primers used for reverse transcription and qPCR were designed according to stem-loop miRNA primer design principle, and the sequences were listed in Table 1. As a common method in similar studies, U6 was used as an internal control to normalize the expression levels of miRNAs. The qPCR experiments were carried out in a BioRad iQ5 system using SYBR Green Master Mix (Takara) following the manufacturer's recommendations, and the data were analyzed based on the $2^{-\Delta\Delta Ct}$ method. Each group has three replicate samples, and all reactions were performed in triplicate.

3. Results

3.1. Overview of miRNA sequencing data

To gain an overview of small RNAs in the gill tissue of the whiteleg shrimp and identify miRNAs potentially involved in hypoxia tolerance, two small RNA libraries were constructed separately from gill tissues under normal condition and exposed to hypoxia challenge, and sequenced with the Illumina platform. For the two small RNA libraries representing the control group (CG) and the stress-challenged group (SG), respectively, a total of 14,499,209 and 16,533,855 raw reads were generated (Table 2). After removing low-quality reads, a total of 13,636,310 and 15,749,755 clean reads were obtained for the CG library and the SG library, respectively. Analysis of length distribution of clean reads showed that small RNAs of 22 nt in length were the most common type, followed by those of 21 nt (Fig. 1).

3.2. Annotation of miRNAs in L. vannamei

Using the BLAST tools, all the clean reads were firstly subjected to sequence alignment against various types of recorded sequences including rRNA, tRNA, miRNA, snRNA, snoRNA, exon, intron, and repeat sequences in major databases such as the GenBank and Rfam database. The number and proportion of sequence tags aligned to different categories of small RNAs are shown in Table 3. Among all the clean reads, miRNAs accounted for 55.85% of the total sequence tags or 5.14% of the unique sequence tags in the CG library, and 56.10% of the total sequence tags or 6.81% of the unique sequence tags in the SG library (Table 3).

To identify miRNAs homologous to known miRNAs in other species, the reads were compared to all animal miRNA sequences recorded in the miRBase database 21.0. The number of unique sequences matched to known miRNA, miRNA-5p or miRNA-3p sequences in the database was summarized in Table 4. In the end, a total of 2109 and 3069 unique miRNAs were identified from the CG and SG libraries, corresponding to 6411 and 8533 homologous miRNA sequences (Table S1) in the database (http://www.mirbase.org/), respectively. 1854 miRNAs were detected in both libraries, and 255 miRNAs were only expressed in the control group while 1215 miRNAs were specifically expressed in the experimental group. In addition, according to the miRNA prediction result, 8 novel miRNAs were identified from the two libraries.

3.3. Differential expression profiles of miRNAs upon hypoxia challenge

By comparing the expression levels of miRNAs between the two samples, differentially expressed miRNAs in response to hypoxia were identified. Using a criterion of $\log_2$fold-change > 1, 1208 annotated miRNAs and 5 novel miRNAs were detected as showing significantly different expression levels. Interestingly, the vast majority of these DEM were actually up-regulated upon hypoxia stress, with 1181 miRNAs showing significant increase in expression level and only 32 miRNAs showing reduced expression. Remarkably, several miRNAs, such as ola-miR-146a-5p and mmu-miR-222-3p, exhibited a very high level of expression induction ($\log_2$fold-change > 10). Among the down-regulated miRNAs, nve-miR-9415 showed the highest fold-change ($\log_2$fold-change $= -4.97$), followed by asu-miR-133-3p ($\log_2$fold-change $= -4.39$) and tca-bantam-5p ($\log_2$fold-change $= -2.62$).

3.4. Target prediction for differently expressed miRNAs and functional enrichment analysis

Using the transcriptome assembly of the whiteleg shrimp as a reference, putative targets of the differently expressed miRNAs were predicted. For the 1208 annotated DEMs, a total of 65,249 transcripts with Nr annotation were detected as having at least one potential miRNA binding site in the 3′ UTR region (Table S2). The majority of the miRNAs were found to have more than one potential target gene, and these target genes were associated with a variety of biological functions. For the most up-regulated miRNAs such as miR-146a-5p and miR-222a-3p, some potential target genes included integrin alpha 5, E3 SUMO-protein ligase RanBP2, cuticular protein, Fushi tarazu factor 1 (FTZ-F1), Histone-lysine N-methyltransferase, Sodium/hydrogen exchanger 9, crustacean hematopoietic factor, and C-type lectin 2. For the most suppressed miRNAs such as miR-133-3p and bantam-5p, some potential target genes included transforming growth factor-beta regulator I, E3 ubiquitin-protein ligase UBR4-like, zinc transporter SLC39A7, juvenile hormone epoxide hydrolase, and Ultrabithorax. To gain an overview of the significantly enriched biological processes and pathways represented by the targets of these differentially expressed miRNAs, GO and KEGG enrichment analysis were further performed. On the top of the most enriched GO term list were “extracellular vesicular exosome” and “plasma membrane” in the category of cellular component, “sequence-specific DNA binding transcription factor

Table 1
Primers used for miRNA expression validation.

ID	Primer sequence	
miR-210	Forward	GCACTGTGCGTGTGACAG
	Reverse	CAGTGCGTGTCGTGGAGT
	RT[a]	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCAGCCG
miR-143	Forward	GCGGTCTGAGATGAAGCACT
	Reverse	CAGTGCGTGTCGTGGAGT
	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGAGCTAC
miR-146b-5p	Forward	GCGCTGAGAACTGAAGTCC
	Reverse	CAGTGCGTGTCGTGGAGT
	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACCCATCTA
miR-222-3p	Forward	GCGAGCTACATCTGGCTACTG
	Reverse	CAGTGCGTGTCGTGGAGT
	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACAGAGACC
miR-21	Forward	GCGCTAGCTTATCAGACTGG
	Reverse	CAGTGCGTGTCGTGGAGT
	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACGCCAACA
miR-10b	Forward	GAACCCTGTGGATCCGAT
	Reverse	CAGTGCGTGTCGTGGAGT
	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACACAAG
miR-133a	Forward	GCGCTTGTCCCCTTCAAC
	Reverse	CAGTGCGTGTCGTGGAGT
	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACAGCTG
miR-1000	Forward	GCGCGATATTGTCCTGTCA
	Reverse	CAGTGCGTGTCGTGGAGT
	RT	GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACACTGCTG
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

[a] RT: reverse transcription.

Table 2
Summary statistics of sequencing data.

Type	Control group	Stress group
Read Length	49	49
Total Raw Reads	14,499,209	16,533,855
Total Raw Bases	724,960,450	826,692,750
Total Clean Reads	13,636,310	15,749,755
Total Clean Reads Ratio(%)	94.05	95.26
Total Clean Bases	306,757,921	355,001,740
Total Clean Bases Ratio(%)	42.31	42.94
Total Short Valid Length Reads	660,360	523,669
Total Short Valid Length Reads Ratio(%)	4.55	3.17
Total Invalid Adapter Reads	131,574	168,231
Total Invalid Adapter Reads Ratio(%)	0.91	1.02

activity" and "poly(A) RNA binding" in the category of molecular function, and "negative regulation of transcription from RNA polymerase II promoter" and "SRP-dependent cotranslational protein targeting to membrane" in the category of biological process (Fig. 2). In addition, KEGG enrichment analysis revealed that many important pathways were significantly over-represented. Notably, these pathways included some related to cytoskeleton remodelling such as "adherens junction" and "regulation of actin cytoskeleton", some related to immune response such as "amoebiasis" and "bacterial invasion of epithelial cells, and some related to signaling transduction such as "signaling pathways regulating pluripotency of stem cells" and "chemokine signaling pathway" (Fig. 3).

3.5. Validation of miRNA expression profile

To validate the expression patterns of miRNAs from deep sequencing result, eight miRNAs showing various expression level changes were randomly selected for stem-loop qPCR validation. The results of qPCR were highly consistent with that of the Illumina sequencing method. Although the absolute levels of expression change estimated by these two methods were not exactly the same, all the validated miRNAs showed comparable expression profiles between the two methods (Fig. 4). These data suggested that analysis of differential miRNA

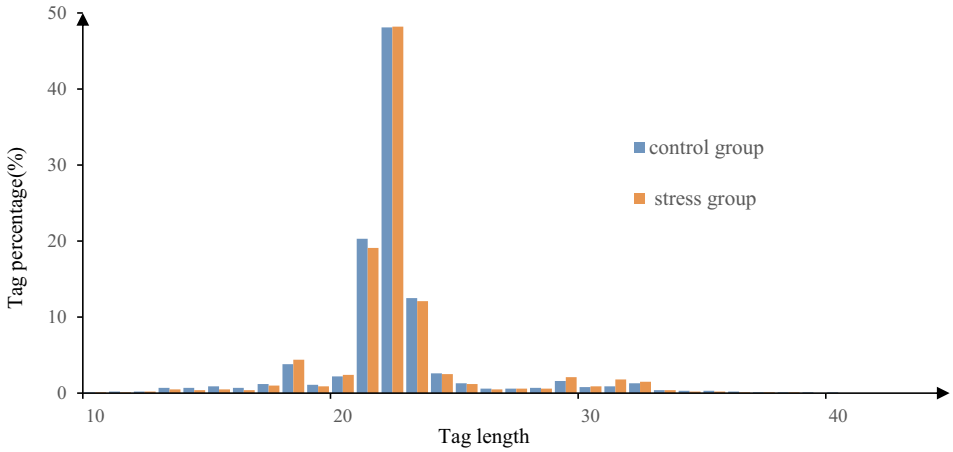

Fig. 1. Length distribution of the clean reads for the two samples. miRNAs of 22 nt in length were the most common type, followed by those of 21 nt.

Table 3
Number of reads matched to various types of sequences.

Category	Control group		Stress group	
	Unique reads (percentage)	Total reads (percentage)	Unique reads (percentage)	Total reads (percentage)
exon_antisense	2393 (0.48%)	859651 (6.30%)	2114 (0.41%)	44825 (0.28%)
exon_sense	965 (0.19%)	4582 (0.03%)	1547 (0.30%)	856798 (5.44%)
intron_antisense	661 (0.13%)	6685 (0.05%)	700 (0.13%)	7150 (0.05%)
intron_sense	576 (0.11%)	2976 (0.02%)	626 (0.12%)	1486 (0.01%)
miRNA	25868 (5.14%)	7615504 (55.85%)	35329 (6.81%)	8835258 (56.10%)
rRNA	63538 (12.62%)	511824 (3.75%)	59868 (11.53%)	646706 (4.11%)
rRNAetc	141 (0.03%)	774 (0.01%)	185 (0.04%)	672 (0.004%)
repeat	1764 (0.35%)	2846 (0.02%)	2494 (0.48%)	4257 (0.03%)
snRNA	9022 (1.79%)	81358 (0.60%)	9413 (1.81%)	79573 (0.51%)
snoRNA	8058 (1.60%)	41724 (0.31%)	9885 (1.90%)	45231 (0.29%)
tRNA	34616 (6.88%)	415185 (3.04%)	40695 (7.84%)	627449 (3.98%)
unannotated	355835 (70.68%)	4093201 (30.02%)	356270 (68.63%)	4600350 (29.21%)

Table 4
Summary of annotation against the miRBase database.

Category	miRBase database	Control group	Stress group
No. of miRNAs	10988	3371	4157
No. of miRNA-5p	7835	1522	2344
No. of miRNA-3p	7888	1518	2032
No. of hairpin	21263	6894	8435
No. of matched miRNA		6411	8533
No. of unique miRNA		2109	3069

expression based on high-throughput sequencing was reliable.

4. Discussion

Hypoxia is a common form of abiotic stressor in aquatic environment [3]. In shrimp farming featured by high cultivation density and frequent environmental changes, hypoxia represented a critical factor limiting the growth and survival rate of the cultured animals [5]. Thus, a considerable amount of effort has been devoted to understand the molecular mechanism of hypoxia tolerance in shrimp including *L. vannamei* [4]. In recent years, accumulating evidence has showed that miRNAs play an important role in the physiological response to stress [15]. Through extensive post-transcriptional mRNA recoding, miRNAs enable living organisms to diversify their transcriptomes and proteomes, allowing them to exhibit more transient phenotypic plasticity in the face of stressful conditions such as hypoxia [32]. However, miRNAs in crustaceans have not been extensively studied, and information on their involvement in hypoxia tolerance is still limited [11]. As the genome sequence of *L. vannamei* is still unavailable, deep sequencing offers a feasible method for effective miRNA identification. Here, through the Illumina HiSeq 2500 high-throughput sequencing

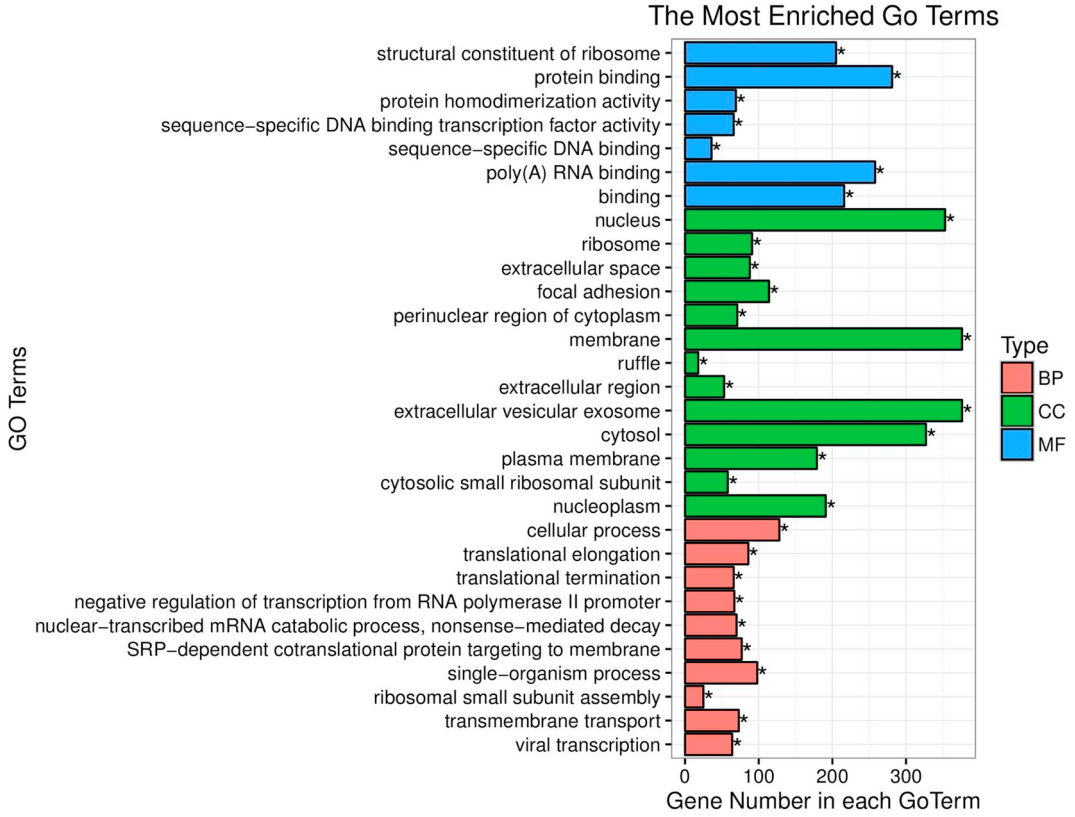

Fig. 2. Gene Ontology enrichment analysis. The most enriched GO terms represented by the putative target genes of the differentially expressed miRNAs were shown. BP, biological process; CC, cellular component; MF, molecular function.

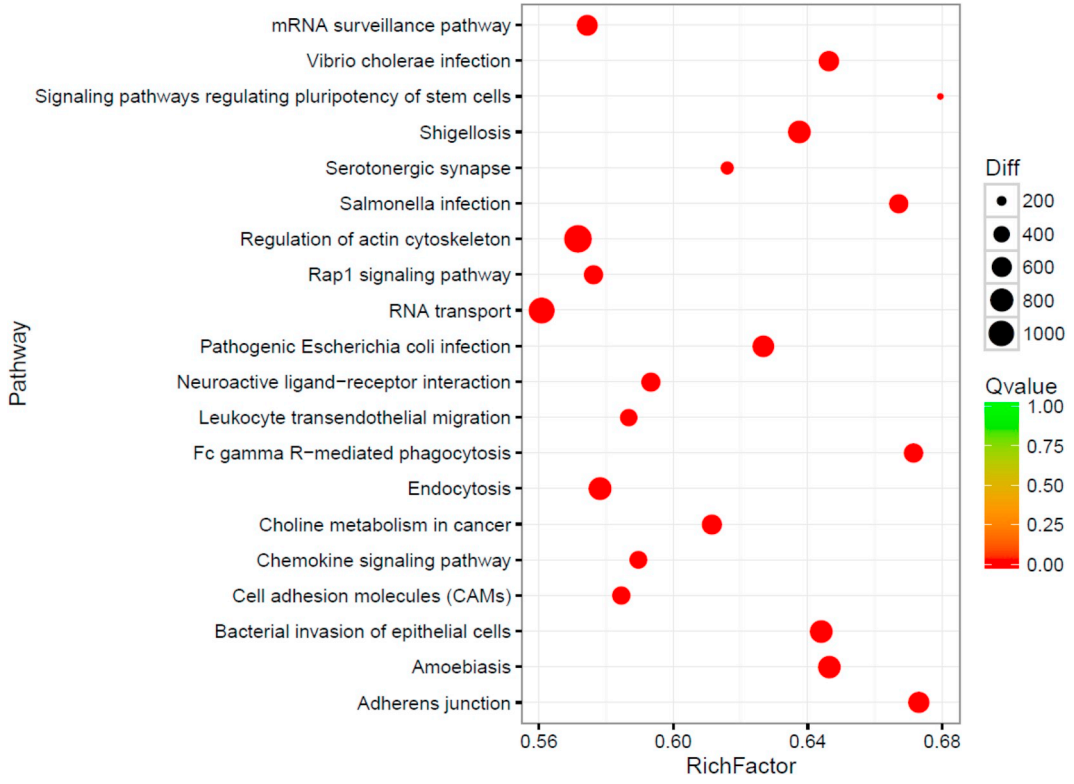

Fig. 3. KEGG pathway enrichment analysis. The most enriched KEGG pathways represented by the putative target genes of the differentially expressed miRNAs were shown. Size of the circle represented number of genes and colors denoted different Q values. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

platform, we surveyed the small RNAs from the gill tissue of *L. vannamei*, as the first step toward understanding the regulatory roles of miRNAs in hypoxia stress response in shrimp.

A total of 3324 known miRNAs and 8 putative novel miRNAs were identified, providing a valuable resource for future investigation on the functional mechanism of miRNAs in shrimp. Compared with previous similar studies on shrimp, the number of miRNAs identified in the present study was significantly larger. This may be partly due to the fact that more miRNAs sequences have been deposited into the miRBase database over the years, providing a better basis for miRNA annotation

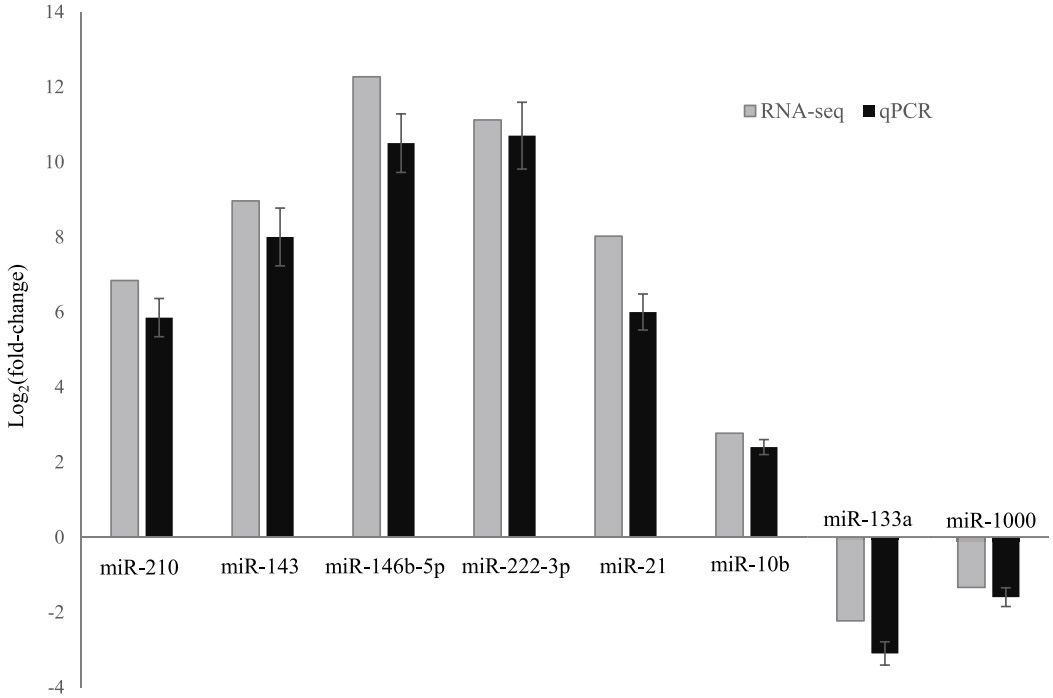

Fig. 4. Comparison of gene expression data between RNA-Seq and qPCR. The eight selected miRNAs showed concordant expression patterns when the two different methods were used.

[23]. Another main reason was many variants or isoforms of the same miRNA family were present in the library. In particular, many of the identified miRNAs had more than one isoform, showing base deletions, addition or base substitutions in the 3′ end. End variation of miRNAs is a frequent phenomenon observed in a wide range of organisms including decapod crustaceans such as *M. nipponense* [11], and our finding was in line with previous reports on miRNA editing [14]. The miRNA isoforms may derive from different gene loci that are under divergent transcriptional regulation [33]. Moreover, variations in the sequence may result in difference in the function of miRNA, affecting its regulatory activity and binding site specificity [11]. While the presence of a large number of miRNA isoforms in *L. vannamei* is suggestive of their potentially diversified roles in shrimp, the functional significance of these miRNA end variations in hypoxia stress response are yet to be demonstrated by experimental validation [14]. In addition, despite the large number of miRNAs identified here, the vast majority (~70%) of the unique sequencing reads actually cannot find similar sequences in the database. This was probably due to the lack of genome data for *L. vannamei* and closely related species [23], and these unannotated small RNA sequences may represent a reservoir of shrimp-specific miRNAs that can be explored in the future when more reference genomes are available.

Comparing the expression levels of miRNAs between the two samples, differentially expressed miRNAs upon hypoxia exposure were identified. 1213 miRNAs were detected as showing differential expression levels and the vast majority of them were up-regulated in response to hypoxia stress. As miRNAs mainly exert their functions through repressing expression of their targets genes [15], up-regulation of the majority of the miRNAs may imply that upon hypoxia stress, the animals would increase the abundancy of most miRNAs probably to inhibit translation of their targets genes [34]. This notion is consistent with previous findings that in stressful conditions, gene transcription and translation activities in the cell are generally suppressed to minimize energy consumption and avoid protein mis-folding [34]. Up-regulation of miRNAs may represent an effective means to inhibit protein synthesis under stressful hypoxia condition [35].

A closer look at the identities of the differentially expressed miRNAs revealed that many previously reported miRNAs involved in hypoxia tolerance of aquatic organisms were also identified in the whiteleg shrimp. For example, miR-210 was a well-known transcriptional target of hypoxia-inducible factor (HIF) [36], and it showed drastic induction in response to hypoxia in various cell types [37]. Similarly, expression of miR-210 which predictably targeted the same genes in shrimp was also found to significantly increase ($\log_2$fold-change > 7.83) in our study, indicating the evolutionarily conserved role of miR-210 in regulating hypoxia stress response across a wide spectrum of species [36]. In addition, several miRNAs belonging to the let-7 gene family, such as let-7a-3p, let-7c and let-7b-3p, also exhibited significant induction upon hypoxia challenge. Previous studies have indicated that the let-7 gene family is actively involved in the immune response to microbial infection by regulating the expression of major cytokine-inducible genes [38,39]. The results of our study may suggest that hypoxia could trigger the coordinated regulation of immune-related gene expression through miRNAs such as let-7. Moreover, miR-143 and miR-101 are two miRNAs that can directly target the key glycolytic enzyme hexokinase [40,41], the first key enzyme of the glycolytic pathway. In our study, both miR-143 and miR-101 were induced by hypoxia. Previous studies in fish has demonstrated that under hypoxic condition, hexokinase could accelerate the rate of glycolysis to produce ATP anaerobically [42]. In L. *vannamei*, it has been reported that hypoxia can induce hexokinase expression in gills with concomitant increase in enzymatic activity, and silencing of the hypoxia inducible factor 1 (HIF-1) blocked the induction of hexokinase in gills but not in muscle, implying tissue-specific post-transcriptional and post-translational regulation of hexokinase [43]. In our study, hexokinase was not a predicted target of miR-143 or miR-101 as in fish, but it was predicted to be targeted by other miRNAs such as miR-252b-5p, miR-24-3p and miR-3966. This may be due to species-specificity and divergent evolution of miRNAs in different lineages, and induction of the miRNAs potentially targeting hexokinase implies that the glycolytic pathway was also likely to be regulated in penaeid shrimp exposed to hypoxia challenge. Other highly conserved miRNAs such as miR-21, miR-252a, miR-17 and miR-29c-3p were also identified as differentially expressed upon hypoxia stress in *L. vannamei*, but information on their functional mechanisms is scarce and further experiments are needed to elucidate their roles in shrimp hypoxia tolerance [11].

In spite of the similarities mentioned above, some hypoxia-responsive miRNAs exhibited contrasting expression patterns in *L. vannamei* when compared with other species. For example, in the oriental river prawn *M. nipponense*, expression of miR-34 quickly dropped to a low level in response to hypoxia treatment [11]. But miR-34 did not show significant expression changes between the control and experiment groups in our study. Another example was miR-133, a well-studied miRNA that has been reported to be involved in the inhibition of neuronal apoptosis and hypoxia stress survival [44]. While its expression remained unchanged in the prawn *M. nipponense* and was significantly induced in the squid *Dosidicus gigas* upon hypoxia exposure [11,32], in our study, apparent down-regulation of miR-133 was observed in the whiteleg shrimp. The divergent expression patterns of these miRNAs may reflect species-specific adaptation to hypoxia, and how they participated in hypoxia stress response of panaeid shrimp requires further investigation.

Analysis of miRNA binding sites in the 3′ UTRs of previously assembled mRNA transcripts revealed that the differentially expressed miRNAs were potentially targeting a wide variety of genes [23], including many with essential roles in hypoxia tolerance. One of these genes was *hypoxia-inducible factor 1 alpha* (*HIF1a*), a predicted target of multiple miRNAs such as miR-150-5p, miR-92a-3p, miR-133, and miR-222b-5p. *HIF1a* functions as a master transcriptional regulator of the adaptive response to hypoxia [45]. Under hypoxic conditions, it activates the transcription of many crucial genes including glucose transporters, glycolytic enzymes, vascular endothelial growth factor, and other genes to facilitate metabolic adaptation to hypoxia [46,47]. The finding that multiple miRNAs can potentially target *HIF1a* in the whiteleg shrimp implied that some of these miRNAs may play important roles in the stress response to hypoxia by post-transcriptional regulation of *HIF1a* expression [17,41]. Other well-known genes that merit further investigation included *heat shock protein 70*, apoptosis-related gene *p53, hypoxia up-regulated protein 1*, *C-type lectin 2*, *crustacean hematopoietic factor*, *ubiquitin-activating enzyme E1*, *NADPH oxidase* and *crustin 2* [29,46,48]. All these genes were potentially targeted by one or more miRNAs. Given the important roles of these protein-coding genes, these miRNAs may contribute to cellular adaptation to hypoxic condition via mediating expression of these genes [49]. Although the interactions between these miRNA-mRNA pairs need to be experimentally validated, miRNAs potentially targeting these crucial genes still represent attractive candidates for follow-up studies on the molecular mechanism of miRNA regulation on hypoxia stress response in shrimp [50–52].

To obtain a deeper insight into which biological processes and metabolic pathways were over-represented, GO and KEGG enrichment analysis of the target genes was further performed. Among the most enriched GO terms, several terms associated with gene transcription and translation process were identified. These terms included “sequence-specific DNA binding transcription factor activity”, “poly(A) RNA binding”, “protein binding”, “translational elongation”, “translational termination”, “negative regulation of transcription from RNA polymerase II promoter”, and “ribosomal small subunit assembly” (Fig. 2). As discussed above, the vast majority of the differentially expressed miRNAs were up-regulated, suggesting that most of their target genes would be repressed and gene translation in general was likely to be constrained under hypoxic condition [34]. Here, enrichment of these

GO terms further supported that gene transcription and translation process was negatively regulated in response to hypoxia [35]. In addition, previous studies on shrimp showed that abiotic stress such as ammonia exposure can trigger dynamic regulation of genes and pathways associated with cytoskeleton remodeling and immune defense [53]. In our study, a number of KEGG pathways related to cytoskeleton remodeling, immune defense and signaling transduction were also significantly enriched when the shrimp were exposed to hypoxia stress (Fig. 3). The result was consistent with previous findings and further demonstrated that cytoskeleton remodeling and immune defense may represent critical components of the cellular stress response (CSR) strategy against hypoxia [53].

5. Conclusion

Overall, the current study illuminates the potential involvement of miRNAs in regulating hypoxia stress response in *L. vannamei*. A large number of hypoxia-responsive miRNAs were identified, including many well-known miRNAs involved in hypoxia tolerance such as miR-210, let-7, miR-143 and miR-101. The vast majority of these miRNAs were up-regulated, suggesting that up-regulation of miRNAs may represent an effective strategy to inhibit protein translation under stressful hypoxia condition. Remarkably, much of hypoxia-induced miRNA changes appeared to be associated with regulation of gene transcription and translation, cytoskeleton remodeling, immune defense and signaling transduction, highlighting the crucial roles of these cellular events in the adaptation of shrimp to hypoxia. The findings deepen our understanding of the biochemical and molecular underpinnings of hypoxia response strategies used by penaeid shrimp, and provide valuable genetic information for following studies on the functional mechanisms of miRNA-mediated post-transcriptional regulation in hypoxia tolerance of shrimp.

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Appendix A. Supplementary data

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