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Identification and analysis of differentially expressed microRNAs in gibel carp *Carassius auratus gibelio* responding to polyinosinic-polycytidylic acid (poly I:C) stimulation

Jialin Zhang [1], Shanling Sun [1], Yiyang Mao, Guo Qiao, Qiang Li [*]

College of Marine and Bioengineering, Yancheng Institute of Technology, Yancheng 224051

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ABSTRACT

Polyinosinic-polycytidylic acid (poly I:C) is a synthesized analogue of viral double-strand RNA and considered as a potential immunostimulant in aquaculture. MicroRNAs (miRNAs) have been reported to play important roles in the development of the immune system and in regulation of host antiviral responses. In our earlier study, it was found that poly I:C pre-treatment could stimulate the resistance against cyprinid herpesvirus 2 (CyHV-2) infection and enhance the antiviral immune response in gibel carp. To understand the role of miRNAs in regulating the host response to poly I:C treatment, we investigated the expression profiles of miRNAs in the head kidney of poly I:C-treated gibel carp with small RNA sequencing technology. When compared with the untreated group, a total of 24 differentially expressed miRNAs were identified in the poly I:C-stimulated fish, among which, 7 and 17 miRNAs were upregulated and downregulated, respectively. Analysis of target genes of these differentially expressed miRNAs found that most targeted mRNAs were involved in catalytic activity, peptidase activity and endopeptidase activity, and were enriched in the metabolic, protein processing in endoplasmic reticulum and oxidative phosphorylation signaling pathways, suggesting that poly I:C could alter the expression of metabolism-related miRNAs in the kidney of gibel carp. Besides, it was noted that some immune-related miRNAs, including inflammation-related miRNAs (miR-192 and miR-731) and interferon-related miRNAs (miR-194a and miR-122), were downregulated after poly I:C treatment. In summary, it was found that poly I:C could regulate the cellular levels of specific miRNAs involved in metabolism and immune responses in the head kidney of gibel carp, which may increase the capacity of the immune cells to fight against pathogens infection.

1. Introduction

Gibel carp, *Carassius auratus gibelio*, is one of the most important popular economic fish species cultured in China. It is artificially cultivated by heterologous sperm stimulating the eggs of crucian carp (Triploid square *carassius auratus* ♀ × Diplont Xingguo red carp ♂) [1]. With rich nutrition and high growth performance, gibel carp has become one of the most cultured fishes in the region of Yangtze River in China, and the annual production reached 2.7 million tons [2]. The fish is characterized by fast growth, large individual size, strong resistance to stress, making it a good material for fish physiology and immunity studies. However, with the rapid development of gibel carp industry, the incidence of various diseases is increasing, and consequently results in serious economic loss. For example, Herpesviral haematopoietic necrosis disease (HVHND) caused by cyprinid herpesvirus 2 (CyHV-2) infection has posed a great threat for the gibel carp industry in China [3]. Unfortunately, there are lack of effective drugs and control measures for this disease until now.

Polyinosinic-polycytidylic acid (poly I:C), a kind of synthesized analogue of viral double-strand RNA, activates the antiviral pattern recognition receptor Toll-like receptor 3 (TLR3), induces the host antiviral response including the secretion of proinflammatory cytokine and activation of type I IFNs [4–5]. Nowadays, poly I:C has been used in maintaining good health and improving disease resistance in fish, making it as a potential immunostimulant and vaccine adjuvant to protect fish from several aquatic virus infection. For instance, poly I:C injection could substantially reduce the cumulative mortality of seven-band grouper (*Epinephelus septemfasciatus*) after viral nervous necrosis

* Corresponding author
E-mail address: fish790608@163.com (Q. Li).
[1] These authors contributed equally to this work.

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(VNN) challenge, and the surviving fish were highly protected from re-challenge [6]. In our earlier study, the efficacy of poly I:C on protecting gibel carp against CyHV-2 infection has been determined, wherein poly I:C treatment decreased the cumulative mortality and induced powerful antiviral immune response [7]. Besides, increasing evidences have demonstrated that poly I:C can be used as a candidate substance for introducing trained innate immunity, which is a newly proposed concept that innate immune defense stimulation could increase nonspecific resistance to infection by homologous or heterologous pathogens [8]. As reported, feeding with poly I:C could induce long-term immune responses against *Edwardsiella piscicida* infection in turbot (*Scophthalmus maximus*) [9].

MicroRNA is a small, highly conserved, non-coding RNA that negatively regulates the target's post-transcriptional gene expression [10–11]. Nowadays, emerging evidences have showed that the host miRNAs are involved in many biological processes including cell proliferation, differentiation, responses to viral infection, and the stress responses [12–13]. Previously, we found that poly I:C pre-treatment could stimulate the resistance against CyHV-2 infection and enhance the antiviral immune response in gibel carp [7]. To further understand the role of poly I:C in regulating the host response, we investigated the expression profiles of miRNAs in the head kidney of poly I:C-treated gibel carp with small RNA sequencing technology. The differentially expressed miRNAs were identified, their target genes were subsequently predicted and their potential functions were annotated. As far as we know, this study describes for the first time the miRNA transcriptomic response to poly I:C stimulation in gibel carp.

2. Materials and methods

2.1. Fish and poly I:C administration

Healthy gibel carp (average weight of 20 ± 2 g) were obtained from a commercial farm located in Yancheng city, Jiangsu province, China. The fish were maintained in the 70 cm × 50 cm × 46 cm fiberglass tanks supplied aerated fresh water under a 12 h:12 h light: dark photoperiod. The average water temperature was kept at room temperature and the oxygen saturation of the water was approximately 8.2 mg/L. The fish were fed once daily with commercial diet at the dose of their 2% weight. After acclimation for 7 days, the fish were randomly distributed into two groups (n=20), the poly I:C group (S group) was intraperitoneally injected with 100 μL of poly I:C (Sigma-Aldrich Co. USA) at a dose of 10 μg/g fish body weight [6], while the control group (D group) was intraperitoneally injected with 100 μL of PBS. At 24 h post-injection (hpi), 5 fish from each group were chosen, anaesthetized with Tricaine methanesulfonate (MS-222), and the head kidney tissues were sampled. To minimize biological variance, all the samples from each group were pooled together and divided equally into 3 replicates as independent replicates. The samples were immediately frozen in liquid nitrogen and stored at −80°C for RNA extraction.

2.2. Small RNA library construction and Illumina sequencing

To construct small RNA libraries, total RNA of each group of head kidney was extracted with TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. RNA purity was checked using the Nanodrop 2000 spectrophotometer (USA), and the concentration was measured in the Qubit 2.0 Fluorometer (Life Technologies, CA, USA). RNA integrity was assessed by the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA). Using the qualified total RNA as input material, sequencing libraries were generated using Small RNA Sample Pre Kit (NEB, USA). Then, the quality and output of the sequencing libraries were evaluated by the Agilent 2100 bioanalyzer and real-time PCR system. Finally, libraries were sequenced on the HiSeq 2000 platform (Illumina Inc., San Diego, CA, USA) at Novogene (TianJin, China) and single-end reads were generated.

2.3. Sequencing data analysis and identification of miRNAs

Raw reads were filtered by removing low-quality reads, reads with 5′ primer contaminants, reads with 3′ primer and reads with poly A. The remaining sequences were trimmed of their adapter sequences and sequences ranged from 18~35 nt in length were chosen. All the high-quality reads were mapped to the *Carassius auratus* (goldfish) genome (Accession No. GCA_003368295.1) reference sequences by Bowtie 2. The fish is a close relative of gibel carp with high genetic similarity, and its genome was used as the reference genome to transfer the annotation. Small RNAs derived from rRNAs, tRNAs, snRNAs and snoRNAs deposited at the Rfam and NCBI GenBank databases were identified by NCBI blast. Identification was performed by comparing the data from two groups against the known maize mature miRNAs and their precursors of all fish in the miRNA database miRbase 20.0. Sequences with perfect matches were identified as known miRNAs, and novel miRNAs were predicted using miREvo and mirdeep 2 software based on the step-loops or hairpin structures.

2.4. Differentially expression of miRNAs

Fold-change and *p*-value was calculated from the normalized expression according the Bayesian method developed by Audic and Claverie. The differentially expressed miRNAs were identified with the screening threshold of p-value < 0.05 [14].

2.5. Validation of MicroRNA expression

The expression level of the DE miRNAs was validated by qRT-PCR. Total RNA of the head kidney from each treatment group was extracted (n = 3 pooled samples, each pooled sample was prepared from five individual samples). RNAs were reverse-transcribed using the Mir-XTM miRNA First-Strand Synthesis Kit (TaKaRa) and qRT-PCR was subsequently performed using the Mir-X miRNA qRT-PCR TB Green Kit (TaKaRa) according to the manufacturer's instructions. U6 RNA was used as an internal standard. All samples were evaluated in triplicate on the CFX96 Real-time PCR Detection System (Bio-Rad, USA), and the relative expression level was calculated with the $2^{-\triangle\triangle Ct}$ method. All the expression data were subjected to a one-way ANOVA, and statistical significance was assumed at $p < 0.05$.

Table 1
Overview of the filtered data.

Sample	total_reads	N% > 10%	low quality	5_adapter_contamine	3_adapter_null or insert_null	with ployA/T/G/C	clean reads
S1	13183812 (100.00%)	406 (0.00%)	151238 (1.15%)	73269 (0.56%)	507761 (3.85%)	8094 (0.06%)	12443044 (94.38%)
S2	13689259 (100.00%)	506 (0.00%)	183090 (1.34%)	81769 (0.60%)	0 (0.00%)	8946 (0.07%)	13414948 (98.00%)
S3	12067459 (100.00%)	1465 (0.01%)	468153 (3.88%)	47953 (0.40%)	243318 (2.02%)	5573 (0.05%)	11300997 (93.65%)
D1	13517921 (100.00%)	558 (0.00%)	192422 (1.42%)	151313 (1.12%)	0 (0.00%)	15610 (0.12%)	13158018 (97.34%)
D2	13690605 (100.00%)	1392 (0.01%)	389641 (2.85%)	91032 (0.66%)	196705 (1.44%)	9350 (0.07%)	13002485 (94.97%)
D3	14229229 (100.00%)	493 (0.00%)	205814 (1.45%)	163621 (1.15%)	1050704 (7.38%)	16334 (0.11%)	12792263 (89.90%)

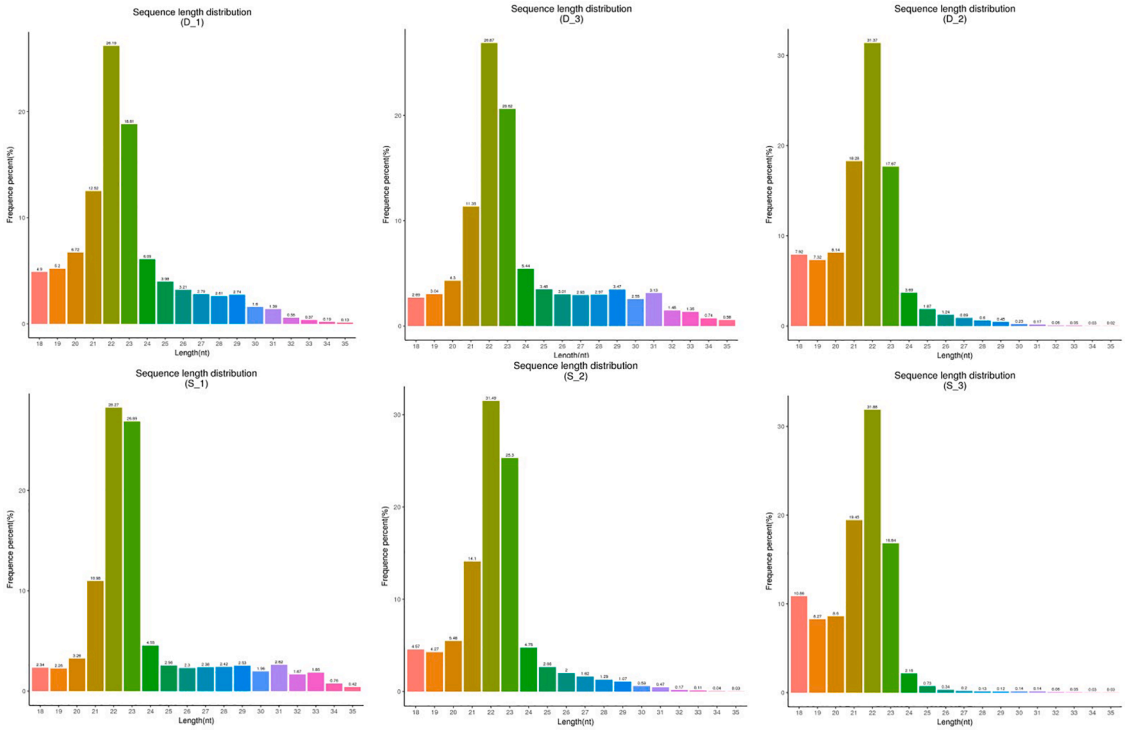

Fig. 1. Length distribution of clean reads.

2.6. *Function analysis of the differentially expressed miRNAs*

The mRNA targets of miRNAs on gibel carp genome were forecasted using MiRanda software with parameters: S≥150 and ΔG≤−30 kcal/moL. The data predicted by both algorithms were combined and the overlaps were considered as the miRNA targets. Furthermore, the

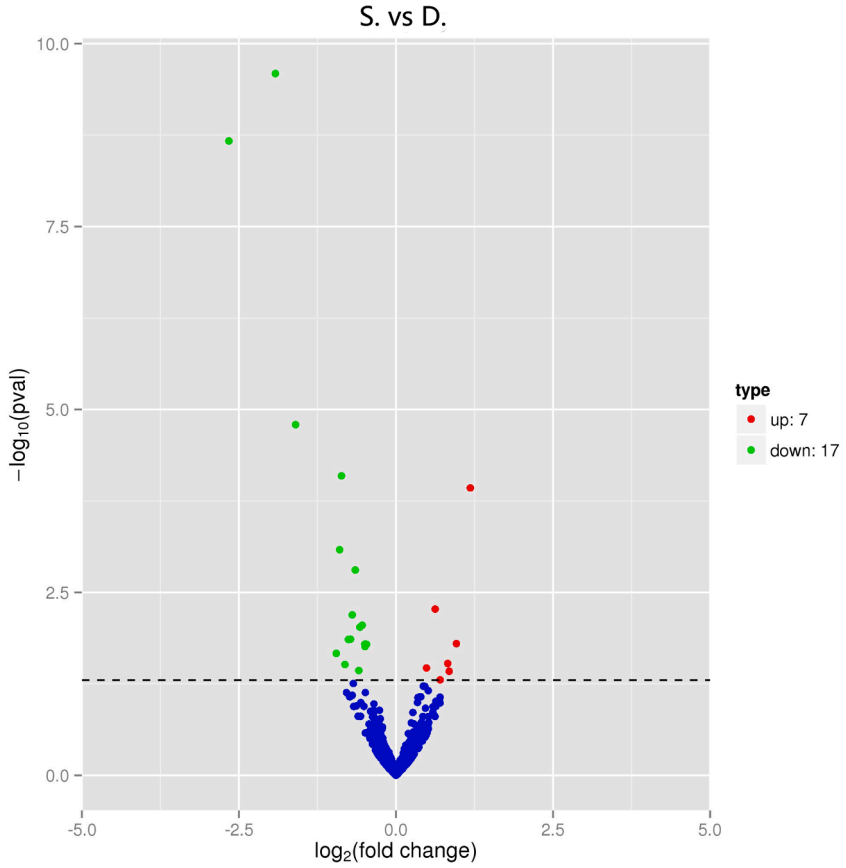

Fig. 2. The "volcano plot" pictures of differentially expressed genes between two groups. S, poly I:C group; D, PBS group.

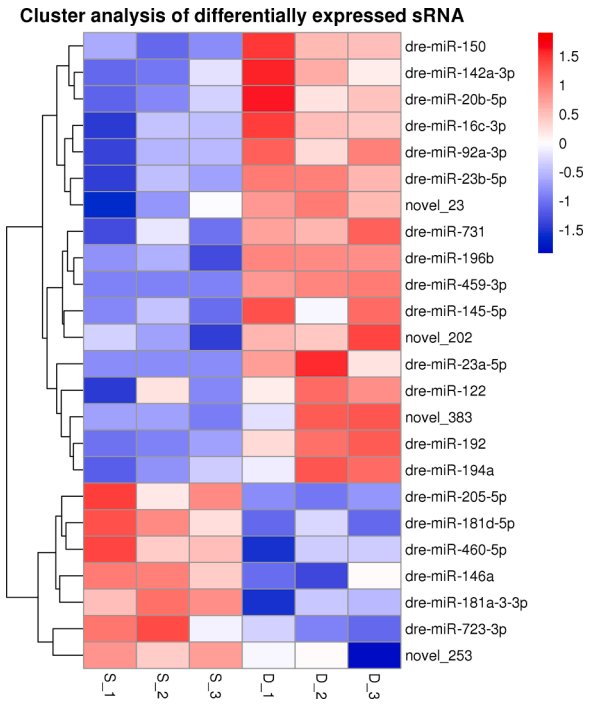

Fig. 3. Cluster analysis of differentially expressed miRNAs. S, poly I:C group; D, PBS group. n = 3.

biological function was annotated with mapping targets genes to each term of the Gene Ontology database using the software DAVID, and the associated signal pathways were determined via a KEGG pathway analysis by the software KOBAS 3.0.

3. Results

3.1. Overview of the high-throughput sequencing data

To investigate the miRNA expression profiles of gibel carp after poly I:C stimulation, small RNA libraries of the head kidney samples from two groups were constructed and sequenced. After quality filtering, about 12~13 million clean reads were generated from each of the three samples (Table 1). The length distribution of the clean reads was analyzed, and a similar trend of the length distribution was observed in the six samples. As shown in Fig. 1, most of the small RNAs were 21-23 nt in length, and 22 nt was the dominant size.

3.2. Identification of miRNA in gibel carp's head kidney

To classify and annotate small RNA sequencing results, the clean reads were aligned with Rfam, cDNA sequences, species repeat library. The tRNAs, snRNAs, rRNAs, and other RNAs were removed and the filtered sequences were aligned with miRBase database. As shown in Table S1, the reads classified into the known miRNAs accounted for 36.7%–56.64% of the total reads, while the novel RNAs accounted for only 0.06%–0.12% of the total unique clean reads by using miRDeep2 software prediction (Table S2).

3.3. Profiles of differentially expressed miRNAs

The differentially-expressed miRNAs, including and novel miRNAs between two groups, were analyzed. The expression of miRNA in two groups was shown by plotting a log2-ratio figure and scatter plot (Fig. 2). A total of 24 differentially expressed miRNAs including 20 known miRNAs and 4 novel miRNAs were significantly differentially expressed ($p<0.05$), including 7 and 17 miRNAs were upregulated and downregulated. As shown in Fig. 3, miR-192, miR-459-3p and miR-194a were down-regulated in the poly I:C group. Of the four novel differentially-expressed miRNAs, novel_383, novel_202 and novel_23 miRNAs were five were downregulated, whereas novel_253 were upregulated. The profiles of differentially expressed miRNAs were shown in Table 2. To confirm those differentially expressed miRNAs between two groups by the sequencing, five DE miRNAs were selected to be verified by qRT-PCR. The results showed that the expression profiles of these DE miRNAs were consistent with those obtained (Fig. 4).

3.4. Target genes prediction and function annotation

To better understand the functions of DE miRNAs of gibel carp, target gene prediction analysis was conducted using miRanda software. In total, 2074 genes were predicted as potential targets of the DE miRNAs that were identified in this study. Subsequently, GO annotation and

Table 2
Profiles of differentially expressed miRNAs.

sRNA	Sequence (5'-3')	S_readcount	D_readcount	log2FoldChange	pval
miR-192	AUGACCUAUGAAUUGACAGCC	335.2857673	1557.371	-1.9189	2.57E-10
miR-459-3p	CAGGGAAUCUCUGUUACUGGGIG	0.42516078	28.9946	-2.6595	2.14E-09
miR-194a	UGUAACAGCAACUCCAUGUGG	116.5116478	484.1212	-1.5981	1.61E-05
miR-150	UCUCCCAAUCCUUGUACCAGUG	56129.97815	106702.5	-0.86647	8.08E-05
miR-205-5p	UCCUUCAUUCCACCGGAGUCUG	173.6053103	67.05123	1.1829	0.000118
miR-145-5p	GUCCAGUUUUCCCAGGAAUCCC	25566.67315	51039.57	-0.89689	0.000826
miR-92a-3p	UAUUGCACUUGUCCCGGCCUGU	76153.65866	122518.2	-0.64767	0.001563
miR-146a	UGAGAACUGAAUUCCAUAGAUGG	226491.6234	142400	0.62413	0.005348
miR-142a-3p	UGUAGUGUUUCCUACUUUAUGGA	273.7438465	464.5156	-0.69594	0.006408
miR-16c-3p	UCCAAUAUUGCUCGUGCUGCUGA	3635.883715	5397.797	-0.53795	0.008881
miR-20b-5p	CAAAGUGCUCACAGUGCAGGUAG	1526.532019	2337.852	-0.5746	0.009429
novel_202	CCGAUUYCUUUUGGUGUUCAGAGU	139.7295229	248.6544	-0.72343	0.013784
miR-196b	UAGGUAGUUUCAAGUUGUUGGG	40.79465644	75.73866	-0.75703	0.01391
miR-723-3p	AAGACAUCAAUUAAAUCUGUGCU	37.54857976	15.10451	0.9614	0.01585
miR-731	AAUGACACGUUUUCUCCCGGAUCG	9926.116722	14288.33	-0.4957	0.016154
novel_23	GCUGGCUAUGGAUUCUGUGUCC	5228.596212	7367.426	-0.46995	0.016182
miR-23b-5p	GGGUUCCUGGCGUGCUGAUUU	229.1196567	329.343	-0.49295	0.017346
novel_383	ACAGGUGUGGAACAUUAAUUCU	0.53416085	7.950229	-0.95084	0.021525
novel_253	CAAAUUAUGAUGUGCUGUCACC	72.24234109	34.31526	0.82388	0.029598
miR-23a-5p	GAAUUCCUGGCAGAGUGAUUU	0.70416021	5.201512	-0.81201	0.030579
miR-460-5p	CCUGCAUUGUACACACUGUGCG	410.392135	285.7737	0.48572	0.034107
miR-122	UGGAGUGUGACAAUGGUGUUUG	526.7047585	837.5674	-0.59185	0.036951
miR-181d-5p	UACAUUCAUUGAUGUCGUUGGGUU	7.808343033	7.0357	0.84621	0.037814
miR-181a-3-3p	ACCAUCGAGUGUUGAGUGUACC	43.4749099	23.58727	0.70256	0.049505

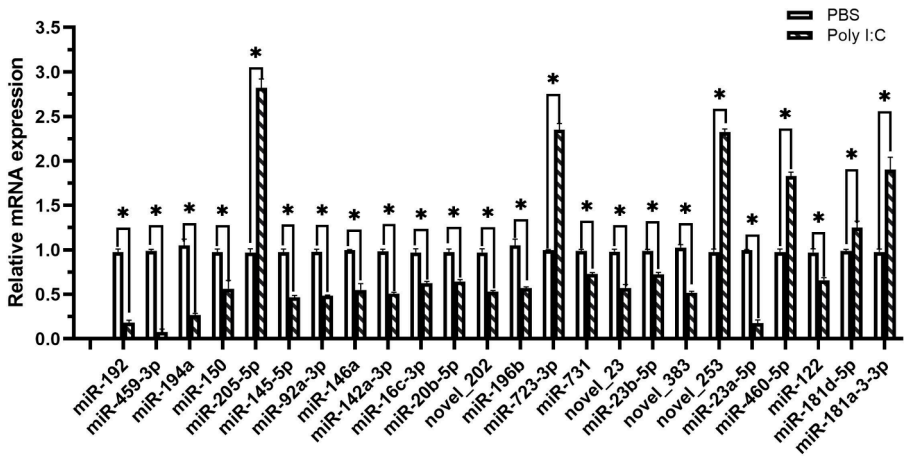

Fig. 4. Validation of relative expression levels of of the DE miRNAs by qRT-PCR. * indicates statistically-significant differences between poly I:C and PBS samples (* $p < 0.05$).

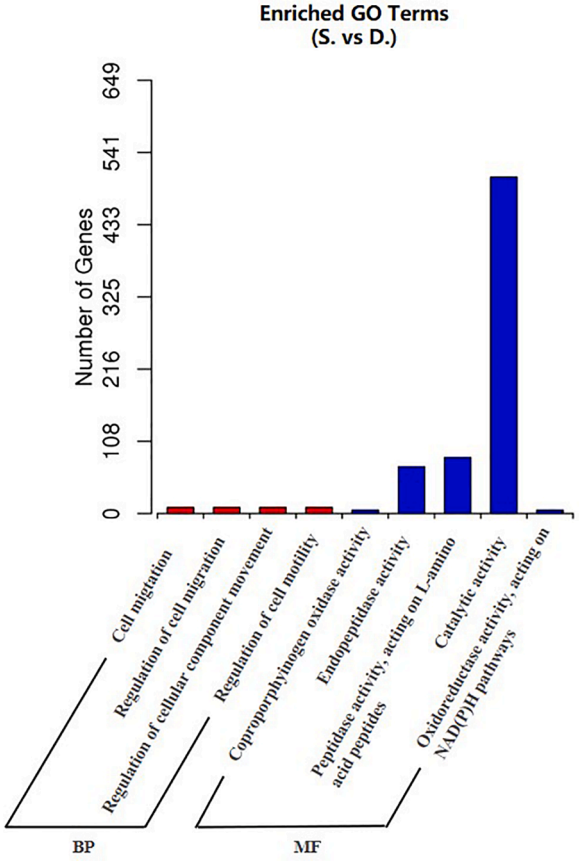

Fig. 5. GO term enrichment for significantly different miRNA.

KEGG pathway analysis were conducted. The GO analysis of identified predicted target genes revealed that most of the target genes were involved in catalytic activity, peptidase activity and endopeptidase activity classes (Fig. 5), suggesting the important role of miRNA in metabolic regulation in gibel carp. KEGG pathway analysis showed that most of the miRNA targets were enriched in metabolic, protein processing in endoplasmic reticulum and oxidative phosphorylation signal pathways (Fig. 6). Besides, it was noted that some immune-related miRNAs, including inflammation-related miRNAs (miR-192 and miR-731) and interferon-related miRNAs (miR-194a and miR-122), were down-regulated after poly I:C treatment.

4. Discussion

Poly I:C is a viral RNA mimic that can induce immune responses similar to that seen during viral infection [15]. For gibel carp, it was found that poly I:C could reduce the mortality of CyHV-2 infection, and improve the mRNA expression profiles of immune-related genes in our previous study [7]. Our present study focused on the miRNA-posttranscriptional regulation in the head kidney of gibel carp after poly I:C treatment. The reason why we chose the tissue is due to the fact that head kidney is the haematopoietic organ in fish, where a large number of immune cells are produced. Thus, head kidney becomes an ideal material for studying the immunogenesis mechanism of fish [16]. Here, we investigated the miRNA transcriptome profiles of head kidney of gibel carp at 24 h post poly I:C stimulation. By high-throughput sequencing, there were 24 differentially expressed miRNAs in head kidney treated with poly I:C, including 7 upregulated miRNAs and 17 downregulated miRNAs. Compared with other miRNA sequencings, the identified differentially expressed miRNAs were less abundant. There are two reasons accountable for this situation: One possible reason is that poly I:C is a small molecule with a simple structure, which has limited biological functions. Another contributing cause is that the total microRNAs mapped on the reference genome was fewer than other studies. Besides, it is noted that the majority of DE miRNAs showed down-regulated expression in head kidney. The down-regulation of these miRNAs could increase the expression of their target genes, which may have positive effects on the host's respond to the poly I:C stimulation.

Emerging evidences have showed that fish can obtain trained innate immunity using ligands to pattern recognition receptors [17]. It offers an interesting and attractive approach to increase disease resistance for fish culture. Poly I:C can activate TLR3 and trigger innate responses, thus making it a promising material for trained innate immunity. Actually, the potential of poly I:C pre-treatment to induce trained immunity has been confirmed. For example, poly I:C could confer protection against a bacterial infection with a Gram-negative pathogen in mice [8]. As reported, intracellular metabolism plays important roles during induction of trained immunity [18]. Metabolism-related signaling cascades including glycolysis, oxidative phosphorylation, the tricarboxylic acid cycle, amino acid, and lipid metabolism has been demonstrated to be interplaying pathways that are crucial for the establishment of innate immune memory [19]. Here, KEGG pathway analysis showed that most of the miRNA targets were enriched in metabolic signal pathways. This is in accordance with a previous report, in which several metabolites were accumulated in poly I:C-treated fish [20]. After poly I:C stimulation, these metabolism-related miRNAs in head kidney were induced and might regulate the intracellular metabolism via their target, thereby

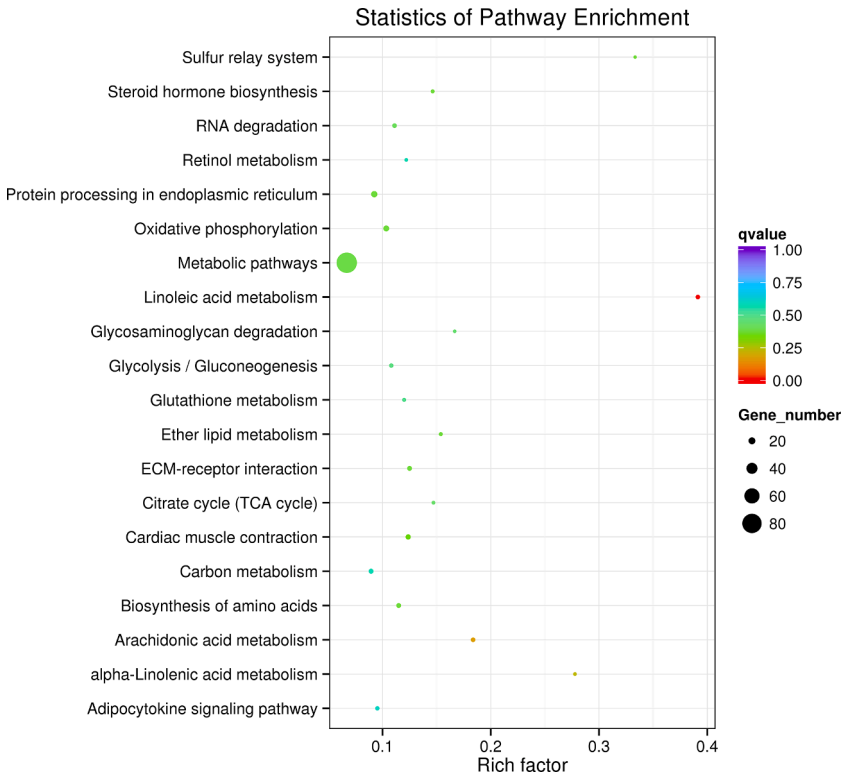

Fig. 6. Pathway classifications of differentially expressed genes according to KEGG results.

contributing to increasing the capacity of the innate immune cells and establishment of innate immune memory.

In fish, interferon (IFN) system could respond to viral infection and induce an "antiviral state", providing an important first line of defense against various viruses [21]. Poly I:C is known to be an immunostimulant that acts as one of the most potent IFN inducers [22]. Here, we found that wo IFN -related miRNAs (miR-194a and miR-122) were differentially expressed in poly I:C group. As reported, miRNA-194a could interact specifically with the 3′UTR of IRF7 in a negative manner, resulting in inhibition of IRF7 expression and blocking the promoter activity of type I interferon [23]. As for miR-122, it could modulate type-I interferon expression by blocking the suppressor of cytokine signaling 1[24]. It was found that the expression of miR-194a and miR-122 were down-regulated in the head kidney of the poly I:C-stimulated gibel carp, suggesting that miR-194a and miR-122 may be promising candidate miRNAs involved in the regulation of type-I interferon system induced by poly I:C.

Previous report revealed that poly I:C could induce inflammation reaction, while appropriate inflammatory response could play a protective role to defend viral infection [25–26]. In this study, we found that two inflammation-related miRNAs (miR-192 and miR-731) were differentially expressed at 24h h after poly I:C treatment. MiR-192 could negatively regulate the expression of its targets gene IL-1RI, resulting in the activation of the signaling cascade to arouse the immune response in reaction against pathogen in Japanese flounder [27]. MiR-731 is known to be involved in the inflammation and apoptosis processes via targeting TLR and apoptosis pathways in grass carp [28]. It was noted that both miR-192 and miR-731 were down-regulated expressed in the head kidney of gibel carp after poly I:C stimulation, which suggested that the two miRNAs may play important roles in mediating inflammation reactions induced by poly I:C stimulation in gibel carp.

5. Conclusion and future perspective

Overall, a total of 24 differentially expressed miRNAs in the head kidney of poly I:C-stimulated gibel carp compared to the control fish, including 17 downregulated miRNAs and 7 upregulated miRNAs. Functional analysis revealed that the majority of DE miRNAs were enriched in metabolism and immune signal pathways, suggesting that poly I:C could regulate the cellular levels of specific miRNAs involved in metabolism and immune responses in the head kidney of gibel carp, which may increase the capacity of the immune cells to fight against pathogens infection. As we know, this is the first report of the miRNA transcriptome of gibel carp in response to poly I:C stimulation. Further work is needed to elucidate the precise role of identified miRNA on immune regulation and the potential for the biomarkers of trained innate immunity.

Ethics statement

All experiments were performed according to the regulation of local and central government of China.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data was used for the research described in the article.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsirep.2023.100083.

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