Functional & Integrative Genomics (2019) 19:265–280
https://doi.org/10.1007/s10142-018-0643-7

ORIGINAL ARTICLE

Analysis of miRNA-seq in the liver of common carp (*Cyprinus carpio* L.) in response to different environmental temperatures

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Received: 3 June 2018 / Revised: 11 September 2018 / Accepted: 25 October 2018 / Published online: 15 November 2018
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Abstract

Water temperature affects the survival, growth, immunity, reproduction, and productivity of farmed fish. The temperature beyond suitable range will disrupt the normal physiological activity. Common carp (*Cyprinus carpio* L.) is a representative eurythermic fish; they are able to sense and respond to changes in water temperature by adjusting their physiology. To investigate the miRNAs in common carp at different temperatures, nine liver small-RNA libraries (5 °C, 17 °C, and 30 °C, each group have three biological repetitions) were constructed and sequenced using high-throughput sequencing. A total of 110 known miRNAs were identified. Twenty-nine known miRNAs were differentially expressed compared with in control group. GO and KEGG analysis indicated that the miRNAs may play important roles in metabolism and environment information processing. Specifically, we considered the insulin-signaling and glycerophospholipid metabolism pathway, and the results show that in 30 °C, miR-301a, miR-203b-5p, and miR-210-3p were upregulated; their target genes which are the mechanistic targets of the rapamycin kinase (*mtor*) gene and the protein kinase AMP-activated catalytic subunit alpha 1 (*prkaa1*) gene in the insulin-signaling pathway were downregulated. And miR-9-5p, miR-27d, miR-92b-3p, and miR-155 were upregulated; their target genes, 1-acylglycerol-3-phosphate O-acyltransferase 3 (*agpat3*), CDP-diacylglycerol-inositol 3-phosphatidyltransferase (*cdipt*), glycerol-3-phosphate acyltransferase mitochondrial (*gpam*), and phosphatidylglycerophosphate synthase 1 (*pgs1*), in glycerophospholipid metabolism pathway were downregulated. But in 5 °C, the situation was opposite. These findings suggest that significant changes occur in energy metabolism and metabolic processes with components of the cell membrane in different temperatures, which significantly advance our understanding of the regulatory mechanisms underlying the physiological change of temperature stress-induced in liver, specifically with regard to miRNAs. These data provide a foundation for further studies of the role of miRNAs in environmental adaptation in fish.

Keywords Eurythermic fish · Illumina sequencing · MicroRNAs · Differential gene expression · Gene Ontology analysis · KEGG analysis · Temperature adaptation

JunLong Sun and LiuLan Zhao contributed equally to this work.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s10142-018-0643-7) contains supplementary material, which is available to authorized users.

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Introduction

The world's average temperature, atmospheric CO_2 concentration, and tropospheric ozone concentration are increasing, thus leading to climate extremes (Intergovernmental Panel on Climate Change 2014). Water temperature is a key environmental factor for fish and is affected by the external climate, and it likewise affects the survival, growth, reproduction, and productivity of farmed fish (López-Olmeda and Sánchez-Vázquez 2011). Most fish species have a relatively low metabolic level and lack insulation and temperature-regulating mechanisms, and they will experience stress if the environmental factors exceed a suitable range, thereby disrupting normal physiological activity and possibly leading to death (Barton 2002; Donaldson et al. 2010). Susceptibility to temperature stress affects the commercial aquaculture of many fishes, such as tilapia (*Oreochromis niloticus*), milkfish (*Chanos chanos*), cobia (*Rachycentron canadum*), and red seabream (*Pagrus major*) (Desaleb et al. 2010; Wu et al. 2011; Hwang et al. 2012). Common carp (*Cyprinus carpio* L.) is a representative eurythermic species now widespread in the world, and farming common carp is an important industry in China (FAO 2016). Losses of common carp due to changes in water temperature are infrequent as these fish are able to sense and respond to the changes through physiological adjustments to the ambient temperature (Wu et al. 2011). Accordingly, many studies have used common carp as an experimental model to investigate temperature tolerance in fish (Gracey et al. 2004; Sun and Liang 2004). To date, research on this topic has mainly focused on the morphological characteristics and related metabolic enzyme activities of the species (Gracey et al. 2004). Aquatic animals can gradually shape their temperature-adaptive phenotypes through extensive biochemical, metabolic, and physiological acclimation processes, which include producing temperature-specific isozymes (Hochachka and Somero 1971), maintaining cell-membrane fluidity (Xu et al. 2015), and synthesizing molecular chaperones (Fader et al. 1994). However, the molecular mechanisms of temperature adaptation in fish remain largely unknown.

With the development of molecular biology and bioinformatics, next-generation sequencing technology (NGST) became ideal for examining temperature adaptation in fishes, as RNA sequencing allows for examination of the entire transcriptome (Wolf 2013). MicroRNAs (miRNAs) are a class of noncoding RNAs, each approximately 22 nucleotides (nt) in length (Bartel and Chen 2004), and they play important roles in the regulation of almost every biological process in eukaryotes (Annibali et al. 2012; Wu et al. 2012). As of 2014, 11,717 mature miRNAs have been identified in mammals, compared to only 1044 in teleost fishes (Bizuayehu and Babiak 2014). MiRNAs interact with the 3′untranslated region (3′UTR) in target mRNAs and reduce protein synthesis via mRNA deadenylation and degradation (Bizuayehu and Babiak 2014; Guo et al. 2010). A single miRNA may modulate hundreds of target genes, while the expression of a single gene may be regulated by multiple miRNAs; thus, almost all genes may be regulated by miRNAs (Miranda et al. 2006). Under stress conditions, miRNA expression is altered as part of the organism's stress response. Identification of the functional role of miRNAs in temperature adaptation has become an important research topic in fish biology, yet the potential effect of ambient water temperatures on the miRNA transcriptome in teleosts has been less reported (Bizuayehu et al. 2015). Therefore, investigations of the molecular mechanisms of temperature adaptation in common carp might advance the scientific research of miRNAs, help decipher the biological basis of the species' response to heat or cold, and make a contribution toward the sustainability of fish culture.

To better understand the temperature adaptation mechanisms of common carp, and especially how they adapt to long-term low-temperature stress (5 °C) or tropical pond waters (30 °C), we compared the miRNA profile in liver of carp under different environmental temperatures to determine which miRNAs are most affected during extended exposure to different temperatures, and which miRNAs show a marked increase in expression at different temperatures (i.e., cold- or heat-responsive miRNAs). Moreover, we predicted the targets of the miRNAs that were differentially expressed at different temperatures, to understand their potential role in the temperature adaptation of common carp.

Methods

Ethics statement

All experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals in China. All experimental procedures and sample collection were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Animal Science and Technology of Sichuan Agricultural University, Sichuan, China, under permit No. DKY-B20121403.

Fish preparation and maintenance

Common carp were obtained from Chengdu Tongwei Aquatic Engineering Technology Research Center, Tong Wei Group Company Ltd. The healthy specimen which an average weight of 500 ± 50 g kept in a recirculating system (200-L tanks with circulating aerated water at 17 °C) to acclimate to our experimental condition for 2 weeks. Fish were satiation feeding with standard diet for carp (31.3% crude protein, 11.6% crude fat, 11.7% ash). Dissolved oxygen (DO) and

temperature were maintained at > 5.8 mg/L (> 80% of air saturation) and 17 °C, respectively.

Experimental design

Ninety fish were randomly divided into three tanks (30 fish in each). For cool and heat stress, all fish were subjected to a stepped cooling regime of 1 °C/h for a maximum of 7 °C/day and warming by the same regime. The fish were cooled from 17 to 5 °C and acclimated at 5 °C which was named low-temperature group (LTG), the other fish were warmed from 17 to 30 °C and acclimated at 30 °C which was named high-temperature group (HTG), and control group (CG) was maintained at 17 °C. The fish were sampled after acclimated for 18 days.

Sample collection and RNA isolation

Sampled after fasting for 2 days, the fish were anesthetized with MS-222. Their livers were collected from three groups in each five fish and immediately snap-frozen in liquid nitrogen, stored at − 80 °C until use. Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and stored at − 80 °C. RNA was quantified and quality was assessed by NanoDrop Spectrophotometer 2000c (NanoDrop Technologies, Wilmington, DE, USA) and 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). Three samples from each group were used for small RNA library construction which OD ratios of 260/280 and 260/230 greater than 1.8 and RNA integrity number (RIN) greater than 8.

Small RNA library construction and sequencing date analysis

Small RNA Cloning Kit (Takara) was used to construct small RNA libraries. RNA was purified by polyacrylamide gelelectrophoresis to enrich for molecules in the 18–32 nt range and then ligated to 5′ and 3′ adapters. The resulting libraries were used as templates for cDNA synthesis followed by PCR amplification and subjected to the Hiseq 2000 sequencing-by-synthesis method. Image analysis, sequencing quality evaluation, and data production summarization were performed using the Illumina/Solexa pipeline. Sequence data were pretreated to discard low-quality reads, reads lacking 3′-adaptors, 5′-adaptor contaminants, and sequences < 18 and > 32 nt. The filtered reads were processed into different read lengths for further analysis. MiRNA gene prediction was conducted using miRDeep2 version 2.0.0.5 program (Friedländer et al., 2008), which allows to determine palindromic sequences around homologous sites and compare the results with the known miRNA genes in the databases. The known miRNAs and novel 3p- and 5p-derived miRNAs were identifed by BLAST searches against specific species precursors in miRBase 21.0 (Kozomara and Griffiths-Jones 2013; Zhang et al. 2016).

Differential miRNA expression analysis

The counts of the identified miRNAs in both libraries were normalized as transcripts per million (TPM). The normalization formula used was as follows: normalized expression = actual miRNA count/total count of clean reads × 10^6. If the normalized expression of a miRNA was zero, it was modified to 0.01 for further analysis, and if the normalized expression of a miRNA for both samples was less than 1, it was removed from the differential expression analysis. The miRNA expression profiles were compared in HTG and LTG with CG. Used edge R with criteria of p value < 0.05 and | log2 (fold-change) normalized | > 1 to determine the differentially expressed of miRNAs.

MiRNA target gene prediction

TargetScan 5.0 (http://www.targetscan.org/) and miRanda 3.3a (http://www.microrna.org/) were used to predict the target genes by differentially expressed miRNAs using *Danio rerio* as a reference. All detected targets with scores and energies less than the threshold parameters of S ≥ 140 (single-residue pair scores) and ΔG ≤ − 7 kcal/mol (minimum free energy) were selected as potential targets (Betel et al. 2010). Finally, the data predicted by both algorithms were combined and the overlaps were calculated. Gene Ontology (GO) (http://www.geneontology.org/) was used to determine the functions of these predicted target genes. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of miRNA's target genes using default settings in Kyoto Encyclopedia of Genes and Genomes (http://www.genome.jp/kegg/). The data were analyzed focusing on environmental information processing and metabolize pathways. Top 20 KEGG pathways passed significance using Bonferroni correction for multiple testing, and then predictive regulation network of major pathways.

Quantitative PCR analysis

Total RNA was used to synthesize mRNA cDNA and miRNA cDNA by using TianGen® FastKing RT Kit (With gDNase) and TianGen® miRcute Plus miRNA First-Strand cDNA Synthesis Kit, respectively. The expression level of target genes and the differentially expressed miRNAs were determined using quantitative PCR (qPCR). The emission intensity was detected by Step One real-time PCR system (Applied Biosystems) under the following steps: initial denaturation step at 95 °C for 20 s, 40 thermal cycling steps consisted of 3 s at 95 °C, 30 s at 60 °C. To adjust for variations in starting template, gene expression was be normalized against β-actin, 18 s rRNA with CG, and then miRNA expression was be

normalized against U6 snRNA with CG. All reactions were run in triplicate and included no template controls for each gene (the reference gene primers are shown in Table S1). MiRNA forward primers were designed based on mature miRNA sequences, and reverse primers were universal primers. The target gene qRT-PCR primers were designed with reference to the relevant known common carp sequences by Primer 5.0. MiRNAs and target gene mRNA were quantified using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak 2008; Livak and Schmittgen 2001), and the level of significance was determined by one-way analysis of variance (ANOVA) with SPSS Statistics 22.0.

Availability of data and materials

All data generated and analyzed during this study are included in this published article. The sequencing raw data has been uploaded to the NCBI_GEO database, and GEO accession is GSE114714.

Results

Overview of the high-throughput sequencing data

To identify miRNAs involved in liver metabolism in common carp under varying temperatures, nine independent small-RNA libraries representing the CG ($n = 3$), LTG ($n = 3$), and HTG ($n = 3$) groups were generated and sequenced (Table 1). Sequencing generated 14,365,460, 14,741,805, and 14,643,040 raw reads from the CG; 13,683,037, 12,787,470, and 12,726,754 raw reads from the HTG; and 13,801,055, 13,319,843, and14,517,656 raw reads from the LTG liver samples, respectively. After read processing and filtering, a total of 13,541,499 (94.26% of the raw reads), 12,739,064 (86.41% of the raw reads), and 10,317,480 (70.46% of the raw reads) high-quality clean reads from CG; 12,585,874 (91.98% of the raw reads), 11,572,759 (90.50% of the raw reads), and 12,292,016 (96.58% of the raw reads) high-quality clean reads from HTG; 12,151,905 (88.05% of the raw reads), 10,593,030 (79.53% of the raw reads), and 13,276,067 (91.45% of the raw reads) high-quality clean reads from LTG, respectively, were selected for further analysis. Most of the small RNAs (sRNAs) in the nine libraries ranged from 21 to 23 nt, and 22 nt was the most common length (Fig. S1). These findings are consistent with similar, previous studies of common carp (Yan et al. 2012), blunt-snout bream (Jiang et al. 2016), and grass carp (Gan et al. 2016).

Identification of known miRNAs

After sRNA annotation and screening, the miRNA sequences in the CG, LTG, and HTG libraries were compared with the known *Cyprinus carpio* L. miRNA sequences listed in miRBase 21. We identified 110 known miRNAs (Table S2) and listed the 10 most abundant known miRNAs in each library (Fig. S2). Notably, the 10 most abundant known miRNAs were similar in the three libraries, accounting for 68.93%, 68.07%, and 67.33% of the known miRNAs in the CG, LTG, and HTG groups, respectively. This finding suggests that these miRNAs are involved in the most basic functions of the carp liver.

Differentially expressed miRNAs in the different temperature groups

First of all, we randomly selected 10 miRNAs to determine their expression patterns using quantitative reverse transcription PCR (RT-qPCR); the miRNA primers are shown in Table S3. The results of RT-qPCR showed significant positive correlation with the RNA-Seq results ($p < 0.05$; Fig. S3). These results showed similar trends with the RNA-Seq results, indicating that our sequencing results were accurate.

In total, 29 miRNAs displayed differential expression in the LTG and HTG as compared with in the CG; of these, 17 were differentially expressed in LTG, and 25 were differentially expressed in HTG. To differentiate the expression of each miRNA and then categorize them, a heatmap was generated and hierarchical clustering was performed using the default settings of the OmicShare Heatmap tools (www.omicshare.com/tools). The cluster results of the 29 differentially expressed miRNAs showed that the CG, LTG, and HTG groups displayed different patterns of expression, and that the HTG and LTG groups had the most similar pattern (Fig. 1); this showed that water temperature could significantly affect the expression of part of miRNAs in the liver of this species.

We subdivided the differentially expressed miRNAs into high-abundance (TPM > 100) and low-abundance (TPM < 100) categories (Table 2). First, we explored the miRNA expression patterns associated with cold tolerance: there were 17 differentially expressed miRNAs in the LTG; among them, five miRNAs were upregulated (two high-abundance miRNAs: miR-27a-3p and miR-27d; three low-abundance miRNAs: miR-135c, miR-203b-5p, and miR-499-5p) and 12 miRNAs were downregulated (four high-abundance miRNAs: miR-122, miR-210-3p, miR-30b, and miR-30d; eight low-abundance miRNAs: miR-155, miR-187, miR-18a, miR-18b-5p, miR-457b-5p, miR-7b, miR-9-5p, and miR-92b-3p). Second, we detected changes in miRNA expression during heat tolerance: 25 miRNAs were differentially expressed in the HTG; among them, seven miRNAs were upregulated (five high-abundance miRNAs: let-7a, miR-10d-5p, miR-128-3p, miR-27a-3p, and miR-27d; two low-abundance miRNAs: miR-489 and miR-499-5p) and 18 miRNAs were downregulated (eight high-abundance

Table 1 Date of the Illumina sequencing for the different temperature groups

	HTG_1	HTG_2	HTG_3	LTG_1	LTG_2	LTG_3	CG_1	CG_2	CG_3
Raw reads	13,683,037	12,787,470	12,726,754	13,801,055	13,319,843	14,517,656	14,365,460	14,741,805	14,643,040
Clean reads	12,585,874	11,572,759	12,292,016	12,151,905	10,593,030	13,276,067	13,541,499	12,739,064	10,317,480
Q30%	95.28	95.09	95.18	95.53	95.02	95.38	95.34	95.39	95.31
Total mapped small RNA	6,832,141	6,166,533	7,497,749	6,743,017	5,567,712	6,155,365	6,085,958	6,429,944	5,741,971
Mapped mature miRNA	110								

miRNAs: miR-122, miR-146a, miR-15b-5p, miR-20a-5p, miR-210-3p, miR-301a, miR-30b, and miR-30d; 10 low-abundance miRNAs: miR-1, miR-155, miR-184, miR-187, miR-18a, miR-18b-5p, miR-203a-3p, miR-457b-5p, miR-459-5p, and miR-9-5p). Third, to understand the involvement of the miRNAs in both cold and heat stress, based on the expression profiles in the liver of carp in the different temperature groups, we found similar miRNA expression profiles in liver for the LTG and HTG groups: that is, three miRNAs (miR-27a-3p, miR-27d, and miR-499-5p) were upregulated in both LTG and HTG, and ten miRNAs (miR-122, miR-210-3p, miR-30b, miR-30d, miR-155, miR-187, miR-18a, miR-18b-5p, miR-457b-5p, and miR-9-5p) were downregulated in both LTG and HTG.

Prediction of miRNA targets in liver of common carp

As only a handful of miRNA targets in fish have been experimentally validated, we used software-based prediction tools to propose the potential target genes using *Danio rerio* as a reference. Therefore, to increase the likelihood of predicting genuine target genes, we considered only targets that were identified by well-established prediction tools to identify the miRNA binding sites. The target genes were identified by overlaps between two databases (TargetScan 5.0 and miRanda 3.3a) through prediction among the 29 differentially expressed miRNAs. Specifically, we identified 6406 genes as the targets for 17 of the CG and LTG differentially expressed miRNAs, and 8509 genes were identified as the targets for 25 of the CG and HTG differentially expressed miRNAs. Furthermore, these potential targets would be subjected to functional annotation and curation.

GO classification analysis

Target genes of differentially expressed miRNAs were annotated with GO classification analysis, using one or more GO term. This analysis provided a hierarchical relationship and more information on the molecular functions (MFs), cellular components (CCs), and biological processes (BPs) in common carp.

GO categorization of the 6406 target genes was predicted by 17 differentially expressed miRNAs between the LTG and CG groups ($p < 0.05$; Fig. S4A). The target genes of the differentially expressed miRNAs were subjected to GO annotation: for CC, the major categories were cell, cell parts, intracellular, and intracellular parts. Genes involved in binding and ion binding were most represented among the MF. For the BP, the major categories were single-organism processes and single-organism cellular processes. Some regulations of biological processes like regulation of the biological process, regulation of cellular processes, cellular responses to stimuli, and

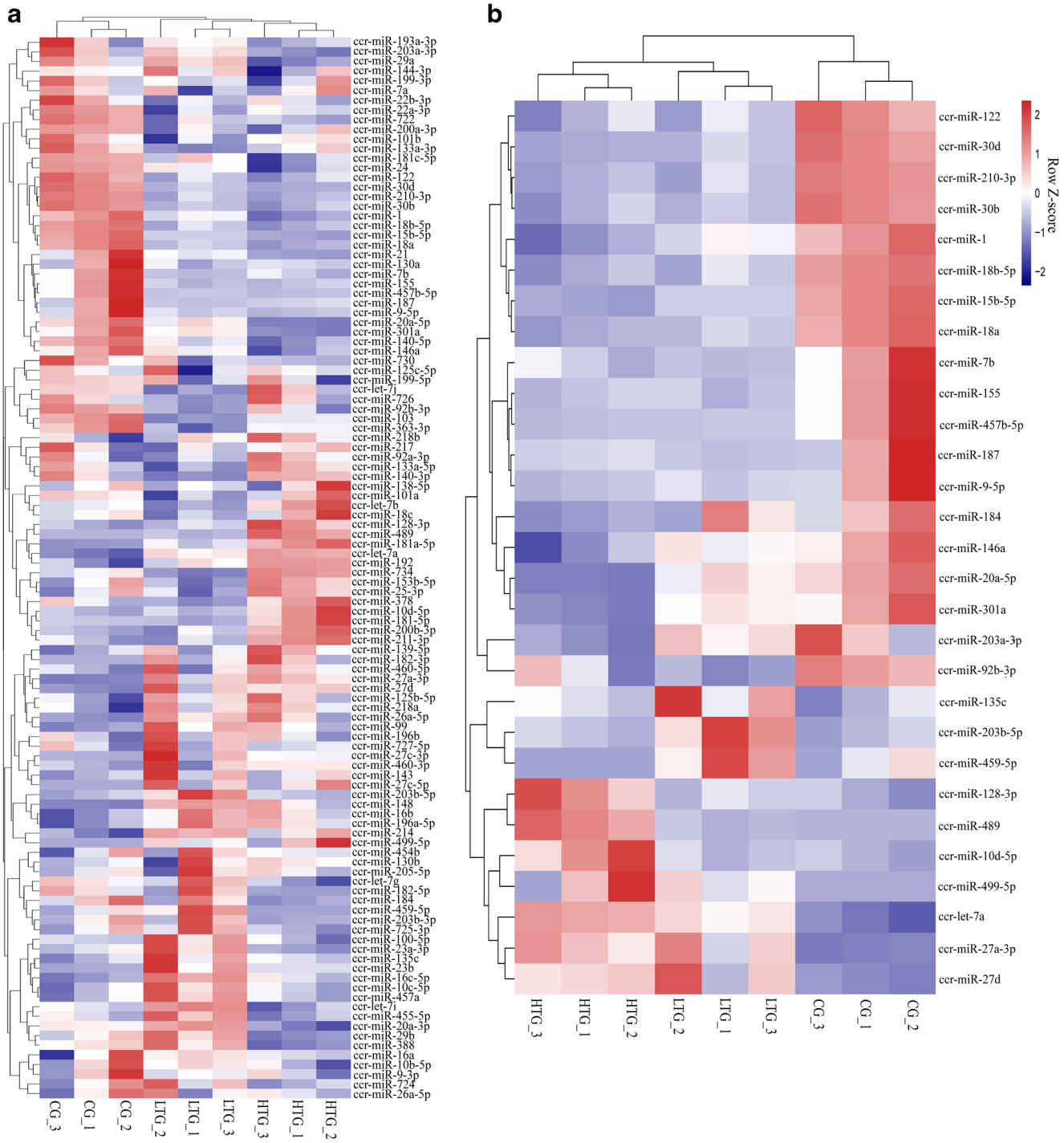

Fig. 1 Heatmap of the conserved miRNAs obtained by sequencing for different temperature groups. **a** Expression profiles of all known miRNAs. **b** Expression profiles of the 29 differentially expressed miRNAs in the HTG and LTG groups, compared with the CG group

single transduction which can experience and regulation of environmental adaptation were enriched.

GO categorization of the 8509 target genes was predicted by 25 differentially expressed miRNAs between the HTG and CG groups ($p < 0.05$; Fig. S4B). Target genes of differentially expressed miRNAs were likewise subjected to GO annotation: for the BP, the major categories were single-organism process, regulation of biological processes, regulation of cellular processes, cellular responses to stimuli, and single transduction were also enriched. Genes involved in binding and ion binding were also most represented among the MF. For the CC, the major categories were cell, cell parts, intracellular, and intracellular parts.

The above results displayed categories both enriched in LTH and HTG were cell, cell part, intracellular and intracellular part of CC, binding and ion binding of MF, single-

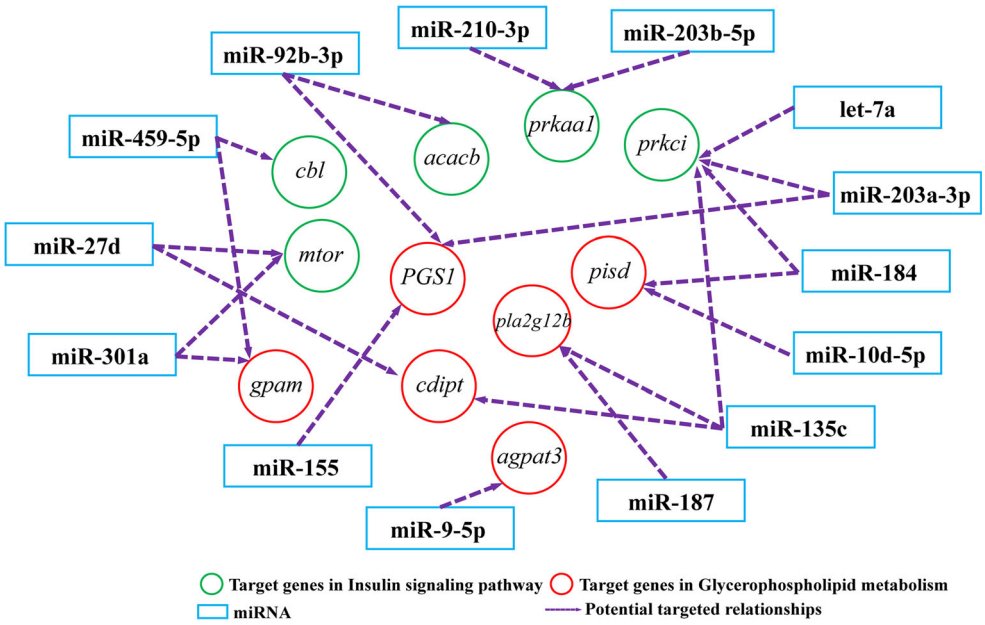

Fig. 2 Predicted target genes for miRNAs in the insulin-signaling pathway and glycerophospholipid metabolism pathways

organism process, regulation of biological process, regulation of cellular process, cellular response to stimulus, and single transduction of BP. These functions must play important roles in the temperature adaptation of common carp. Interestingly, some categories (single-organism metabolic process, small molecule metabolic process, lipid metabolic process, and other cellular component organization or biogenesis, and metabolic process) were also enriched, suggesting a regulation role in the temperature adaptation of common carp.

KEGG classification analysis

A KEGG pathway analysis was also performed for the differentially expressed target genes to identify the biochemical pathways operating in *Cyprinus carpio* L. The results classified 3100 target genes into 160 different pathways between the LTG and CG groups. Similarly, 4173 target genes were classified into 160 different pathways from 25 differentially expressed miRNAs between the HTG and CG groups. Among them, the metabolism and environmental-information-processing pathway categories contained the largest number of target genes in the different temperature groups (Table S4).

Target genes of 17 differentially expressed miRNAs fell into 12 major subgroups between the LTG and CG groups and were involved in several metabolism pathways (such as lipid metabolism, carbohydrate metabolism, amino acid metabolism, glycan biosynthesis and metabolism, and nucleotide metabolism) and three major subgroups involved in environmental-information-processing pathways (namely, signal transduction, signaling molecules and interaction, and cell-membrane transport) (Fig. S5A), but the target genes showed less enrichment than the HTG. Seven pathways showed a significant degree of enrichment ($Q < 0.05$; Fig. S5B), including the insulin-signaling pathway and gonadotropin-releasing hormone (GnRH) signaling pathway in the endocrine system of the organismal system, glycerophospholipid metabolism in lipid metabolism, endocytosis in cellular processes, and the FoxO signaling pathway and ErbB signaling pathway in environmental information processing.

Target genes of 25 differentially expressed miRNAs between the HTG and CG groups fell into 12 major subgroups involved in metabolism pathways (lipid metabolism, carbohydrate metabolism, amino acid metabolism, glycan biosynthesis and metabolism, and nucleotide metabolism) and three major subgroups involved in environmental information processing (signal transduction, signaling molecules and interaction, and cell-membrane transport) (Fig. S5C). Eleven pathways showed strong enrichment ($Q < 0.05$; Fig. S5D), including the insulin-signaling pathway in the endocrine system of the organismal system, glycerophospholipid metabolism in lipid metabolism, focal adhesion and endocytosis in cellular processes, and ErbB signaling pathway, FoxO signaling pathway, mTOR signaling pathway, Notch signaling pathway, MAPK signaling pathway, and ECM-receptor interaction in environmental information processing.

In short, based on the miRNAs' target genes of common carp in different temperature groups, we found similar pathways in the liver in the LTG and HTG groups (Fig. S6), such as the insulin-signaling pathway in the endocrine system, glycerophospholipid metabolism in lipid metabolism, endocytosis, FoxO signaling pathway, and ErbB signaling pathway in signal transduction, which play important roles in sensing, intracellular transduction of stress signals, and maintenance of cell stability of common carp subject to environmental temperature changes.

Table 2 Differentially expressed miRNAs divided into high- and low-abundance categories

High-abundance miRNAs						Low-abundance miRNAs					
miRNA	CG (TPM)	LTG (TPM)	log2(fc)	*p* value	FDR	miRNA	CG (TPM)	LTG (TPM)	log2(fc)	*p* value	FDR
miRNAs with lower expression in the LTG											
ccr-miR-122	42,525.13	19,954.69	− 1.09	1.72E−06	2.70E−05	ccr-miR-155	169.88	64.36	− 1.40	1.05E−03	6.78E−03
ccr-miR-210-3p	4093.91	1230.03	− 1.73	6.00E−10	3.06E−08	ccr-miR-187	23.54	5.06	− 2.22	2.15E−04	1.69E−03
ccr-miR-30b	2714.06	1354.41	− 1.00	2.00E−07	5.49E−06	ccr-miR-18a	93.73	40.32	− 1.22	2.05E−03	1.12E−02
ccr-miR-30d	6741.79	2764.32	− 1.29	0.00E+00	4.80E−09	ccr-miR-18b-5p	28.09	13.45	− 1.06	8.95E−03	3.65E−02
						ccr-miR-457b-5p	101.15	48.77	− 1.05	1.32E−02	4.54E−02
						ccr-miR-7b	2.87	1.17	− 1.29	1.42E−02	4.60E−02
						ccr-miR-9-5p	69.70	22.94	− 1.60	1.68E−03	9.71E−03
						ccr-miR-92b-3p	169.94	66.87	− 1.35	4.42E−04	3.24E−03
miRNAs with higher expression in the LTG											
ccr-miR-27a-3p	283.25	776.43	1.45	6.94E−08	2.54E−06	ccr-miR-135c	2.08	5.12	1.30	1.11E−02	4.21E−02
ccr-miR-27d	206.35	502.78	1.28	2.08E−04	1.69E−03	ccr-miR-203b-5p	0.71	3.16	2.15	1.44E−04	1.44E−03
						ccr-miR-499-5p	3.52	8.79	1.32	1.43E−03	8.73E−03
miRNA	CG(TPM)	HTG(TPM)	log2(fc)	*p* value	FDR	miRNA	CG(TPM)	HTG(TPM)	log2(fc)	*p* value	FDR
miRNAs with lower expression in the HTG											
ccr-miR-122	42,525.13	18,119.84	− 1.23	9.18E−07	8.41E−06	ccr-miR-1	2.19	0.64	− 1.79	2.96E−03	1.05E−02
ccr-miR-146a	45,226.87	16,513.29	− 1.45	6.35E−06	4.11E−05	ccr-miR-155	169.88	66.26	− 1.36	1.09E−03	5.02E−03
ccr-miR-15b-5p	2688.18	1130.29	− 1.25	0.00E+00	0.00E+00	ccr-miR-184	226.97	61.29	− 1.89	2.39E−06	1.88E−05
ccr-miR-20a-5p	1776.45	692.62	− 1.36	0.00E+00	0.00E+00	ccr-miR-187	23.54	6.84	− 1.78	3.92E−03	1.31E−02
ccr-miR-210-3p	4093.91	984.95	− 2.06	0.00E+00	0.00E+00	ccr-miR-18a	93.73	30.94	− 1.60	1.15E−04	6.05E−04
ccr-miR-301a	971.44	318.53	− 1.61	0.00E+00	0.00E+00	ccr-miR-18b-5p	28.09	11.39	− 1.30	1.98E−03	7.50E−03
ccr-miR-30b	2714.06	1250.31	− 1.12	5.50E−09	6.09E−08	ccr-miR-203a-3p	256.60	57.69	− 2.15	2.46E−05	1.42E−04
ccr-miR-30d	6741.79	2229.59	− 1.60	0.00E + 00	0.00E + 00	ccr-miR-457b-5p	101.15	46.14	− 1.13	7.49E−03	2.11E−02
						ccr-miR-459-5p	51.73	3.74	− 3.79	6.28E−06	4.11E−05
						ccr-miR-9-5p	69.70	17.00	− 2.04	1.93E−04	9.63E−04
miRNAs with higher expression in the HTG											
ccr-let-7a	12,455.16	26,523.24	1.09	0.00E+00	1.00E−10	ccr-miR-489	10.39	24.17	1.22	9.41E−04	4.50E−03
ccr-miR-1 0d-5p	129.65	317.72	1.29	1.27E−06	1.07E−05	ccr-miR-499-5p	3.52	12.45	1.82	1.84E−03	7.22E−03
ccr-miR-128-3p	1694.17	3916.78	1.21	1.00E−10	8.00E−10						
ccr-miR-27a-3p	283.25	840.06	1.57	0.00E+00	0.00E+00						
ccr-miR-27d	206.35	478.86	1.21	6.00E−10	7.60E−09						

The data in the table is the average of TPM from three biological replications corresponding to each group

Predicting the regulation function of key miRNAs

This study focused on the pathways of carbohydrate and lipid metabolism; thus, we further analyzed the pathways of insulin signaling and glycerophospholipid metabolism. We screened target genes for miRNAs involved in the insulin signaling pathway and the glycerophospholipid metabolic pathway from known genes in common carp (Fig. 2). There were 10 miRNAs with five target genes in the insulin signaling, and 10 miRNAs with six target genes in glycerophospholipid metabolism. The relative expression of miRNAs is shown in Fig. S7.

To predict their regulatory relationships, we used RT-qPCR to determine relative expression levels. The miRNAs and mRNA primers are shown in Tables S3 and S5, and the results of RT-qPCR showing the expression of potential target gene mRNA are given in Fig. S8. A total of 11 target genes were selected for tests of relative expression; most of the genes showed a higher degree of expression in LTG and a lower degree of expression in HTG as compared with expression in CG. Only the acetyl-CoA carboxylase beta (*acacb*) gene displayed significant downregulation in both the LTG and HTG groups, but the relative expression in LTG was still higher than that in HTG.

Using the mRNA and miRNA expression data (Fig. 3), relative comparisons showed that the phosphatidylglycerophosphate synthase 1 (*pgs1*) gene may negatively regulated by miR-92b-3p and miR-155; miR-9-5p may significantly negatively regulated the 1-acylglycerol-3-phosphate O-acyltransferase 3 (*agpat3*) gene, the CDP-diacylglycerol-inositol 3-phosphatidyltransferase (*cdipt*), and glycerol-3-phosphate acyltransferase; mitochondrial(*gpam*) genes may negatively regulated by miR-27d; and the *gpam* gene may also negatively regulated by miR-301a in glycerophospholipid metabolism pathways. Similarly, the mechanistic target of the rapamycin kinase (*mtor*) gene and the protein kinase AMP-activated catalytic subunit alpha 1 (*prkaa1*) gene of insulin-signaling pathway probably targeted with miR-301a, miR-203b-5p, and miR-210-3p, respectively (with a significantly negative correlation between the miRNA and target mRNA; $p < 0.05$). These results were used to predict a network of regulatory relationships between the miRNAs and their target genes (Fig. 4).

Discussion

Adaptation of common carp to varying temperatures

Global warming and climate change have led to climate extremes (Intergovernmental Panel on Climate Change 2014). In fish, the adaptation to environmental temperatures is the result

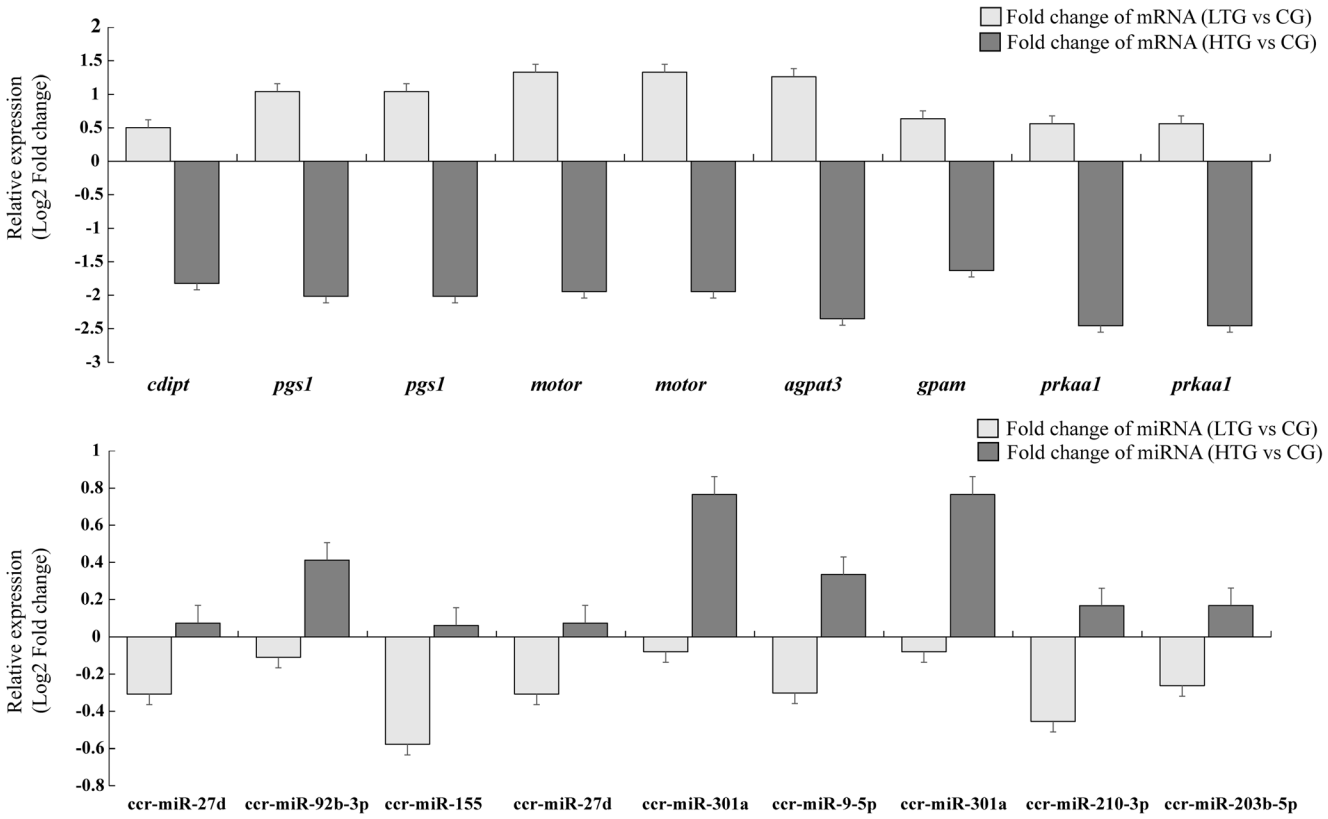

Fig. 3 Comparison of expression patterns of miRNA-mRNA interaction pairs using qRT-PCR

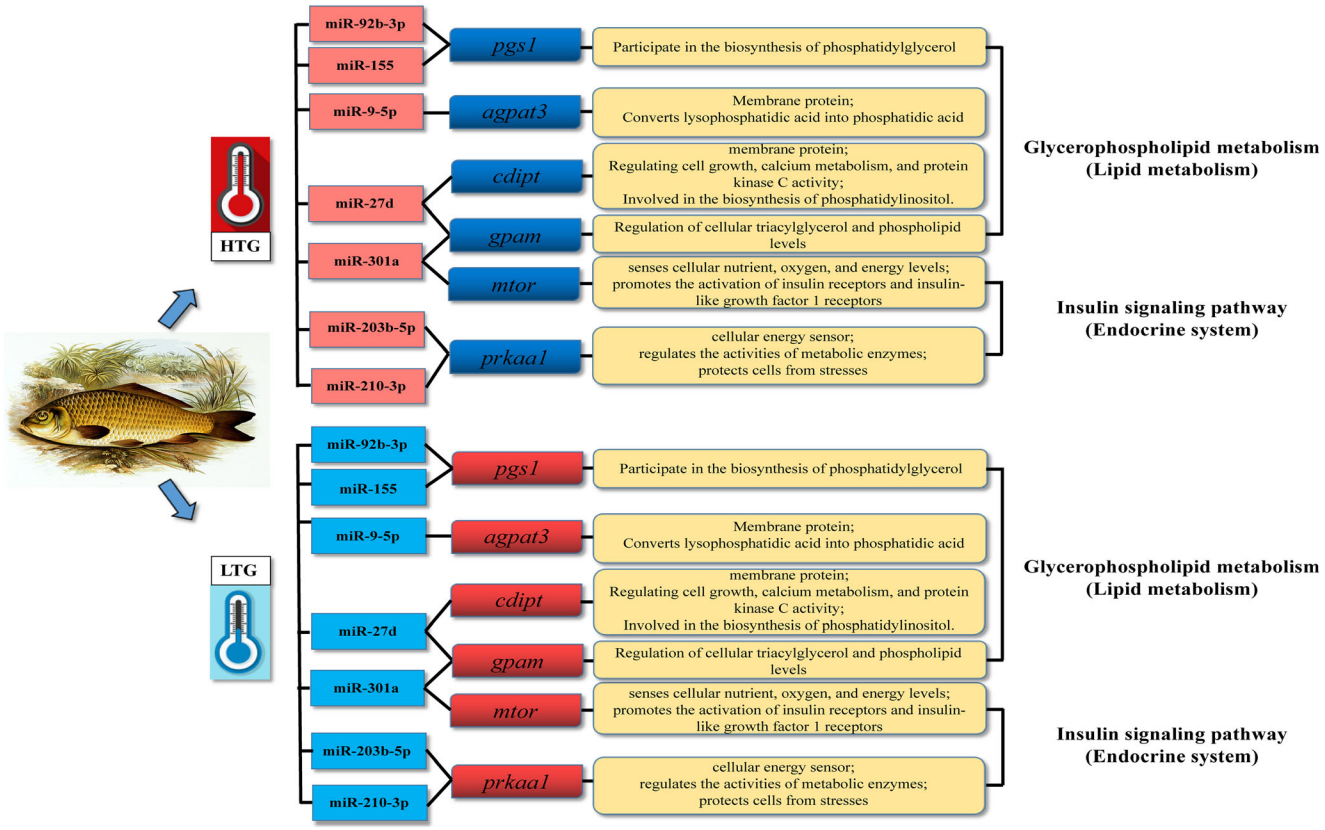

Fig. 4 Potential functional network regulated by miRNAs in the HTG and LTG groups in liver of common carp in response to heat and cold stress. Blue boxes indicated that expression of miRNA or target genes were downregulated. Red boxes indicated that expression of miRNA or target genes were upregulated. The function of the genes were described in the yellow box

of long-term evolution. Thermal tolerance is an especially attractive trait in aquaculture species, and recent experimental evidence suggests that some fish species carry sufficient genetic variation to adapt to different temperatures under strong selection (Barrett et al. 2011). Researchers have characterized the transcriptional responses elicited by cold stress in a number of fish species, including the zebrafish (Chou et al. 2008; Long et al. 2012), channel catfish (Ju et al. 2002), rainbow trout (Vornanen et al. 2005), gilthead seabream (Mininni et al. 2014), Antarctic notothenioid (Chen et al. 2008), as well as common carp (Gracey et al. 2004). The transcriptome responses to heat stress in the nucleated red blood cells of rainbow trout demonstrated that genes involved in the stress response, immune response, and apoptosis presented the highest dysregulation during both early and late transcriptional regulation (Lewis et al. 2010). Such studies have identified a large number of temperature-adaptation regulated genes involved in a variety of biological processes associated with acclimation to both daily and seasonal temperature variations. Common carp in China experience serious cold stress during the winter in northern areas and heat stress during the summer in southern areas; however, the species has adjusted to this temperature range. Previous studies have shown that common carp can endure a wide range of thermal change, including daily temperature cycles with a range of ≥ 20 °C (Wu et al. 2011). Common carp are one of the most representative eurythermic fishes, yet the mechanisms of their temperature adaptation are not clear. Previous researchers have concluded that maintaining cell stability, regulating metabolism, immunity, and cell apoptosis are the main means whereby fish have adapted to fluctuating temperatures. In this study, the energy metabolism of common carp during the process of adapting to temperature change was the main focus, particularly the pathways of carbohydrate metabolism and lipid metabolism. As a major metabolic organ, we selected the liver to study the adaptive mechanisms. In liver, the adaptive significance of these changes may be to provide the lipid and cholesterol building blocks necessary for the extensive restructuring of membranes reported in cold-exposed organisms (Hazel and Williams 1990). Studies of carp have shown significant changes in the liver a few days before the temperature changes, which showed a transition to lipid metabolism (Gracey et al. 2004). Our findings are similar to the results of studies mentioned above and indicate that the liver is indeed an important organ in the process of temperature adaptation in a eurythermic fish.

Molecular pathways in liver that assist with temperature adaptation

We investigated the molecular pathways and cellular functions of target genes that likely characterize the common carp's response to cold or heat stress. The data showed that the enriched target genes were related to signal transduction, cell communication, lipid metabolism, carbohydrate metabolism, and other pathways, implicating these processes in regulation of the temperature-related stress response of common carp. Metabolism and environmental-information-processing pathways that are related to lipid metabolism, carbohydrate metabolism and signal transduction, cellular processes related to cell growth and death, the circulatory system, and the immune system were strongly represented. The new information on differentially expressed miRNAs and their target genes, and the analysis of annotations from the GO and KEGG databases, are a valuable genomic data resource for common carp and will allow further study of the molecular basis of the species' temperature adaptation and energy system. We identified a series of target genes related to ECM-receptor interaction, Notch signaling pathway, mTOR signaling pathway, ErbB signaling pathway, and MAPK signaling pathway. The results are similar to the findings of a study of zebrafish (Ju et al. 2002). In that study, the target genes were related to focal pathways of environmental information processing, through increased mitochondrial functioning and changes in the cell-membrane composition, allowing the fish to adapt to cold or heat stress; thus, we inferred that these pathways probably play significant roles in the temperature response of carp. Similar studies of physiological adaptation in aquatic ectotherms (Eliason and Farrell 2011; Pörtner and Knust 2007; Pörtner 2002) indicated that intraspecific thermal tolerance is set by limitations to aerobic performance, specifically the upper limit of the heart rate to deliver oxygen to tissues; this is due to the temperature-dependent oxygen limitation in aquatic environments, a theory that has been well supported by investigations of many organisms (Pörtner 2010).

We found representation of two mutual KEGG pathways in the genes regulated by cold and heat stress; the insulin signaling pathway and glycerophospholipid metabolism may play essential roles in the adaption of common carp to varying temperatures. The insulin transduction pathway mainly controls the balance of glucose regulation in the body. When insulin increases, it reduces glucose in the liver and transforms glucose into fat to maintain the balance of glucose in the blood (Khan and Pessin 2002). This pathway is also affected by states of fasting, stress levels, and a variety of hormones. When carbohydrates are digested and absorbed, blood glucose levels rise significantly; next, the pancreas senses the increased glucose concentration in the blood, causing a release of insulin that stimulates muscle and other tissues to uptake glucose from the blood stream. When insulin binds to insulin receptors, it causes a cascade of cellular processes to facilitate the cells' storage of glucose (Garvey 2004). Recent studies have supported the role of adipose tissue and inflammation in insulin signaling pathways; Hotamisligil et al. (1992) first showed that inflammatory mediators are involved in insulin-signaling transduction pathways. Hence, the insulin-signaling pathways may participate in the regulation of carbohydrate metabolism and inflammation of common carp under different temperatures. "Homeoviscous adaptation" is a prominent hypothesis concerning the mechanism of temperature adaption and postulates that fish can regulate membrane phospholipid saturation to regulate the fluidity of lipid bilayer (Tiku et al. 1996). Glycerol is the backbone of the fundamental phospholipids used as the self-assembling units of lipid membranes. Phospholipids are essential components of biological membranes; as structural and functional units of membranes, membrane phospholipids ensure normal morphology and functioning of cells. We found that glycerophospholipid metabolism, which is associated with cell-membrane lipids metabolism, was significantly enriched at low and high temperatures. Hsieh and Kuo (2005) found that low temperatures led to the regulation of cell membrane fluidity, where transducers detected changes in the fluidity of the membrane, and then transmitted information, ultimately leading to altered gene expression. The change of cell-membrane-lipid metabolism might alter intercellular substance transportation. Under different temperature conditions (here, 5 °C or 30 °C), common carp are able to adapt to changes in ambient temperature by signaling changes in endocytosis, FoxO signaling pathway, ErbB signaling pathway, insulin signaling pathway, and the glycerophospholipid metabolism participate in the metabolism of cell membrane components; this might affect the exchange of substances between cells.. These functions may play important roles in the sensing, intracellular transduction of stress signals, and maintaining the cell stability of *Cyprinus carpio* L. in response to temperature change.

The regulatory role of miRNAs in temperature adaptation

RNA-Seq is extremely useful in identifying the molecules involved in the response of aquatic vertebrates to a number of environmental factors (Zhang et al. 2013). MiRNA profiles have proven to be extremely valuable in unraveling aspects of the regulation of biological functions (Ambros 2004). In the present study, we used RNA-Seq to analyze the transcriptome-level response to cold (5 °C) or heat (30 °C) stress in common carp. The results revealed 29 differentially expressed miRNAs involved in the processes of a temperature-related stress response, energy metabolism, substance metabolism, and immune response. Here, the potential roles of all the investigated miRNAs were inferred from known and putative targets generalized from other species. We obtained the potential

functional network regulated by miRNAs in the HTG and LTG groups in liver of common carp in response to heat and cold stress (Fig. 4). MiRNAs were considered as sentinels of the cellular stress response as (1) they are posttranscriptional gene regulators, potentially functioning as "quick responders" to cellular stress; (2) as miRNAs regulate numerous targets, they have the capacity to potently and efficiently coordinate a stress response involving numerous genes; (3) owing to their small size and high stability, miRNAs may be less susceptible to certain types of stress (Babar et al. 2008).

In this study, miR-210-3p in liver of common carp showed a significant decrease in response to a low temperature and an increase in response to a high environmental temperature. This result was similar to mammals (Yu et al. 2011). Consequently, miR-210-3p in liver may act to promote the *prkaa1* gene in response to low temperatures, but inhibition of the gene in response to high temperatures. The protein encoded by this gene belongs to the ser/thr protein kinase family. It is the catalytic subunit of the 5′-prime-AMP-activated protein kinase (AMPK). AMPK is a cellular energy sensor, conserved in all eukaryotic cells. The kinase activity of AMPK is activated by the stimuli that increase the cellular AMP/ATP ratio. AMPK regulates the activities of a number of key metabolic enzymes through phosphorylation; it protects cells from stresses that cause ATP depletion by switching off ATP-consuming biosynthetic pathways (Krishan et al. 2014). In vertebrate studies, miR-210 is reported to be implicated in the response to hypoxia, as its expression is increased following hypoxia in human (Mathew and Simon 2009; Huang et al. 2010). miR-210 has been shown to repress mitochondrial respiration by targeting transcripts coding for iron-sulfur cluster scaffold proteins (ISCU1/2) involved in an electron transport function and mitochondrial reduction-oxidation reactions (Favaro et al. 2010), a transcription factor previously shown to be upregulated during anoxia (Larade and Storey 2002). MiR-210 is truly a multifaceted regulator of many cellular functions, such as angiogenesis, cell cycle regulation, DNA damage repair, and regulation of mitochondrial metabolism. The temperature changes could lead to a change in gas exchange efficiency. Therefore, miR-210-3p in liver might act to regulate mitochondrial respiration and associated downstream functions in response to low and high temperatures.

The protein encoded by the *mtor* gene is a member of the phosphatidylinositol 3-kinase-related family of protein kinases (Mitra et al. 2015). mTOR functions as a serine/threonine protein kinase that regulates cell growth, cell proliferation, cell motility, cell survival, protein synthesis, autophagy, and transcription (Hay and Sonenberg 2004). As a core component of mTORC2, mTOR also functions as a tyrosine protein kinase that promotes the activation of insulin receptors and insulin-like growth factor 1 receptors (Yin et al. 2016). miR-301 is a significant negative regulator of the *mtor* gene, and miR-301a may act to promote this gene in liver in response to low temperatures but inhibit the gene in response to high temperatures. Thus, miR-301a in liver may act to regulate sensing of cell nutrients, oxygen, energy levels, and activation of insulin receptors and insulin-like growth factor 1 receptors, all in response to low and high temperatures.

The association between miRNA-14 and lipid metabolism was first found in the fruit fly (*Drosophila melanogaster*) (Xu et al. 2003); since then, numerous other studies have shown that miRNA is involved in lipid metabolism regulation, including in zebrafish (*Danio rerio*) (Her et al. 2011), rainbow trout (*Oncorhynchus mykiss*) (Mennigen et al. 2014a, b), and blunt-snout bream (*Megalobrama amblycephala*) (Zhang et al. 2014).

In our study, we found that miR-92b-3p, miR-155, miR-203b-5p, miR-9-5p, and miR-27d were involved in glycerophospholipid metabolism of lipid metabolism. MiR-92b-3p and miR-155 negatively regulated the *pgs1* gene; the protein encoded by this gene is phosphatidylglycerophosphate synthase 1, which is tightly associated with the cytoplasmic membrane. A similar activity has been reported to be associated with mitochondrial membranes in *Saccharomyces cerevisiae* and higher eukaryotic cells (Dowhan and Hirabayashi 1992). Genetic studies have established that the phosphatidylglycerophosphate synthase is the only enzyme catalyzing the committed step to phosphatidylglycerol synthesis, and that this gene product is essential to cell growth (Lykidis 2007). MiR-203b-5p negatively regulated the *pisd* gene; the protein encoded by this gene is phosphatidylserine decarboxylase, which is the major enzyme of phosphatidylethanolamine synthesis in most types of cells, and it plays a central role in phospholipid metabolism in bacteria to humans. Their evolutionary conservation suggests that these enzymes fulfill a central role in lipid metabolism and membrane biogenesis (Tasseva et al. 2013). No apoptotic cells were detected in the *pisd* knockdown cells; however, knocking out the *pisd* gene results in an increase in mitochondrial membrane potential and a decrease in the oxygen consumption rate. The lack of phosphatidylserine decarboxylase can inhibit the activity of respiratory and electron transport chains (Tasseva et al. 2013). MiR-9-5p negatively regulated the *agpat3* gene; AGPAT3 appears to negatively regulate the formation of Golgi membrane tubules, which likely serve as trafficking intermediates for plasma membrane delivery and for maintenance of an intact Golgi ribbon to influence membrane curvature and effector-protein recruitment (Schmidt et al. 2010). MiR-27d negatively regulated the *cdipt* and *gpam* genes; CDIPT (CDP-diacylglycerol–inositol 3-phosphatidyltransferase) found on the cytoplasmic side of the endoplasmic reticulum and the Golgi apparatus perform the last step in the de novo biosynthesis of phosphatidylinositol (PtdIns) by catalyzing the condensation of cytidine diphosphate-diacylglycerol and myoinositol to produce PtdIns and cytidine monophosphate (CMP) (Paulus and Kennedy

1960; Antonsson 1997). As an important lipid, PtdIns participates in essential metabolic processes, in all plants and animals, directly or via a number of metabolites. The metabolism and biosynthesis of PtdIns are of considerable interest due to its phosphorylated derivatives in energy metabolism, fatty acid metabolism pathways, and intracellular signal transduction in eukaryotic cells (Ansell et al. 1973). Breakdown products of PtdIns are ubiquitous second messengers that function downstream of many G protein-coupled receptors and tyrosine kinases regulating cell growth, protein kinase C activity, and calcium metabolism (Noh et al. 1995). The *gpam* gene is a member of the GPAT gene family (Roy et al. 2002; Roy et al. 2006). The protein enzyme from this gene, mitochondrial glycerol-3-phosphate acyltransferase, catalyzes the first committed step in triglyceride and phospholipid biosynthesis (Brockmöller et al. 2012). Several studies strongly suggested that *gpam* plays a vital role in the metabolism of triglyceride (Roy et al. 2005). Hence, *gpam* likely plays a pivotal role in regulating the cellular levels of triacylglycerol and phospholipid.

Previous reports have indicated that both miRNA-122 and let-7a play an important role in regulating metabolism. For example, miR-122 inhibition the expression of several lipogenic genes led to a significant improvement in liver steatosis in obese mice (Esau et al. 2006; Mennigen et al. 2014a, b). Overexpression of gankyrin activated its expression and thereby induced liver steatosis in zebrafish (Mennigen et al. 2014a, b). MiRNA-122 is also a liver-specific miRNA that controls lipid metabolism in teleosts (Pasquinelli et al. 2000). Let-7 family members are highly conserved and are expressed ubiquitously (Roush, and Slack 2008); the disorder of its expression can inhibit cell differentiation and cause cell disease (Bernstein et al. 2013). Zhang et al. (2014) also found that miR-122 and let-7a were the most abundant miRNAs in liver of blunt-snout bream. In our study, both were significantly expressed under different groups. Taken together, these findings suggest that miR-122 and let-7a are critical regulators of fundamental biological processes in liver of common carp under the low and high temperatures.

Conclusions

We have reported a comprehensive miRNA profile and target-gene dataset for common carp. This work represented an initial foray into the importance of miRNAs in response to cold and heat stress in a teleost species. The miRNAs identified and the target genes annotated provide a valuable genomic resource that extends our understanding of the unique biological characteristics of common carp. This study has shown that signal transduction, cellular component organization or biogenesis, energy production processes, and signal transduction were the most highly enriched pathways under high- and low-temperature stress. Predicted from the target genes of differentially expressed miRNAs at different temperature, all these pathways can be assigned to the following biological functions in common carp, a species that tolerates an exceptionally wide range of water temperatures: signal responses to cold or heat stress, lipid metabolism, a circulatory system which can regulate and effect subsist, immunity and circulatory of fish. More exactly, miRNAs play a significant role in the molecular response of common carp to cold and heat stress by regulating signal transduction, cellular component organization, and various metabolic processes to maintain their survival. In our study, the genes associated with the insulin-signaling pathway and glycerophospholipid metabolism pathway were shown to be significantly downregulated at high temperatures and significantly upregulated at low temperatures; this reveals that significant changes occur in the energy metabolism and metabolic processes associated with cell membrane components of carp at different temperatures. Under low-temperature conditions, the carp might regulate the composition of cell membranes by upregulating genes involved in phospholipid metabolism. Under high-temperature conditions, the carp might regulate the composition of cell membranes by downregulating genes associated with phospholipid metabolism. Accordingly, these miRNAs related to lipid metabolism play an essential role in the regulation of cell membrane fluidity at different ambient water temperatures.

To our knowledge, this study is the first to systematically analysis the miRNAs that respond to different temperatures in liver of common carp. Furthermore, a negative correlation between the expression of miRNAs and their predicted gene targets was identified. Although relatively little information is currently available concerning the biological function of miRNAs identified to date, we strongly suggest that miRNAs play an important role in modulating gene expression involved in the physiological response to temperature stress in the fish liver.

Acknowledgments We are grateful to JiDeng Ma, Long Jin, and KeRen Long at Sichuan Agriculture University for technical support and software operation, and we gratefully acknowledge the assistance of Qing Xiao and Jie Du with fish rearing and tissue sampling. We thank Harry Taylor, PhD, from Liwen Bianji, Edanz Editing China (www.liwenbianji.cn/ac), for editing the English text of a draft of this manuscript.

Author contributions Junlong Sun, Song Yang, and Mingzhou Li conceived and designed the experiments; Hao Wu and Wenqiang Lian performed the experiments; Junlong Sun and Song Yang performed data analysis; Can Cui, Zongjun Du, and Wei Luo contributed to sample collection; Junlong Sun wrote the paper; Liulan Zhao assisted with writing and proofreading.

Funding This research was supported by the Double Support Project fund of the Sichuan Agricultural University (SICAU, No. 03572406).

Compliance with ethical standards

Conflicts of interest The authors declare that they have no conflict interest.

Consent for publication Not applicable.

Ethics approval and consent to participate All experiments were performed according to the Guidelines for the Care and Use of Laboratory Animals in China. All experimental procedures and sample collection were approved by the Institutional Animal Care and Use Committee (IACUC) of the College of Animal Science and Technology of Sichuan Agricultural University, Sichuan, China, under permit No. DKY-B20121403.

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