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Identification and Characterization of MicroRNAs in the Spleen of Common Carp Immune Organ

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ABSTRACT

MicroRNAs (miRNAs) play an important role in the regulation of many fundamental biological processes in eukaryotes; however, miRNAs associated with immune functions in the common carp have not been reported. In this study, a small-RNA cDNA library was constructed from the spleen of the common carp. A total of 10,603,456 high-quality clean reads, representing 293,603 unique sequences, were obtained from the small-cDNA library using the Solexa sequencing. By the bioinformatic analysis, 194 conserved miRNAs and 12 novel miRNAs were identified in the carp spleen. The abundant miRNAs principally belong to 30 miRNA gene families such as let-7, mir-10, mir-15, mir-30, and so on. The conservation analysis showed that 23 families were present both in protostomes and deuterostomes, 46 families were conserved only in vertebrates, and 5 families (mir-430, mir-722, mir-724, mir-734, and mir-738) were identified only in fish species. Furthermore, GO enrichment analysis and KEGG pathway analysis suggested that miRNAs expressed in the spleen of common carp are involved in immune system development, lymphoid organ development, lymphocyte activation, immune response, B cell receptor signaling pathway, T cell receptor signaling pathway, Fc gamma R-mediated phagocytosis, Toll-like receptor signaling pathway, and so on. This study described the miRNA transcriptome in spleen tissue for the first time in the common carp. The results expand the number of known common carp miRNAs and provides a meaningful framework to understand the common carp immune system and defense mechanisms. J. Cell. Biochem. 115: 1768–1778, 2014.

KEY WORDS: MicroRNA; SPLEEN; EXPRESSION PROFILES; SOLEXA SEQUENCING; COMMON CARP

The common carp (*Cyprinus Carpio L.*) is one of the main commercial fishes captured and cultured worldwide. The production of this species, however, is effected by its high disease susceptibility. Fish kept under the intensive culture conditions are constantly exposed to a wide range of stressors. Any stressor that exceeds the ability of fish to adapt may be lethal or will facilitate the infection by opportunistic pathogens present in the water [Singh et al., 2012]. Under these circumstances, fish depend heavily on innate or non-specific immune responses to rapid elimination of pathogens [Camp et al., 2000]. Therefore, an important approach to disease prevention is to culture strains of fish with enhanced resistance to major diseases using molecular breeding methods [Singh et al., 2012]. However, molecular techniques are still not widely used in the breeding of the common carp due to a lack of genetic and genomic information. To date, studies about the disease resistant traits of the common carp are still in its infancy, and many disease resistant traits have not been well defined by molecular markers.

MicroRNAs (miRNAs) are endogenous small (~22 nt) non-protein-coding RNAs. They exist in a wide range of invertebrates and vertebrates and play essential roles in gene expression regulation [Sontheimer and Carthew, 2005]. Many studies have demonstrated that miRNA is critical for proper immune system development and also plays an important role in a variety of other aspects of the immune system. For example, a lack of Dicer leads to lower diversity and survival of B cells [Koralov et al., 2008]. miR-155

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null mice exhibit greatly impaired immune responses upon immunization, suggesting that this miRNA may be involved in regulating T helper cell differentiation and the germinal center T cell dependent antibody response [Thai et al., 2007]. MicroRNA expression in the common carp has been characterized in several studies; however, none have focused on their expression in the immune organs. An earlier study by Yan et al. [2012] identified 188 conserved miRNAs and seven novel miRNAs from the skeletal muscle of 1-year-old common carp. Some miRNAs (e.g., miR-1, miR-133a-3p, and miR-206) were specifically expressed in muscle-containing organs [Yan et al., 2012], suggesting that these miRNAs may have specific functions in the process of skeletal muscle development of the common carp. Another study by Zhu et al. [2012] also identified 92 conserved miRNAs and 21 common carp-specific miRNAs from the mixed tissue sample of 17 adult common carp using homology-based prediction combined with small RNA sequencing. Hierarchical clustering of the RT-qPCR products showed that the expression patterns of some of the miRNA families were associated with certain development stages of common carp [Zhu et al., 2012]. These miRNA profiling studies indicate that miRNA plays an important role in regulating development of common carp. However, much remains about the role of miRNA in immune development and function have not been investigated in common carp.

The spleen is regarded as a major immune organ in fish. Obtaining a profile of the miRNAs that are expressed in the spleen should facilitate our understanding of the involvement of miRNA in immune system functions. Therefore, in the present study, we constructed a small-RNA cDNA library from the spleen of common carp. Through high throughput sequencing of the small RNA library and subsequent bioinformatic analysis, miRNAs in the spleen of common carp were identified.

MATERIALS AND METHODS

EXPERIMENTAL ANIMALS, TISSUE COLLECTION, AND RNA ISOLATION

All animal experiments were performed using cultivated *Cyprinus carpio haematopterus Temminck* in the Henan Agricultural University in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of China. The study was approved by the Ethics Committee of the Freshwater Fisheries Research Institute of Henan Province. The spleen was collected by dissection from 1- and 2-year-old common carp, snap-frozen in liquid nitrogen, and stored at −80°C until analysis. Total RNAs from spleen tissues were extracted using TRIzol reagent (Takara). Tissues were collected separately from three individuals at each developmental stage. After the quality of RNA was assessed by electrophoresis and ultraviolet spectrophotometry, equal concentrations of RNAs from the different individual spleen tissues were pooled together.

SMALL RNA LIBRARY CONSTRUCTION AND HIGH THROUGHPUT SEQUENCING

Total RNA used for the generation of the small RNA library were obtained from six individual spleen tissues (i.e., 1- and 2-year-old fish, three individual per stage). Small RNAs of 16–30 nt in length were first isolated from the total RNA by size fractionation in a 15% TBE urea polyacrylamide gel, and these small RNAs were ligated with the 3′ and 5′ adapter ligation. Then reverse transcription PCR using the RT primer was used to create cDNA constructs, and PCR reactions were performed using the forward and reverse primers. Subsequently, the PCR product was purified and sequenced by the BGI-Shenzhen using Solexa technology. The image files generated by the sequencer were processed to produce digital-quality data. After masking of adaptor sequences and removal of contaminated reads, clean reads were processed for computational analysis.

SEQUENCE ANALYSIS AND IDENTIFICATION OF miRNAs

Initial reads obtained from Solexa sequencing were processed by summarizing data production, evaluating sequencing quality, calculating the length distribution of small RNA reads, removing low quality reads and adaptor sequences. After elimination of redundancy, sequences $\geq$ 18 nt were used for subsequent analyses.

The clean reads were blasted against the Rfam database (http://www.sanger.ac.uk/software/Rfam) and the GenBank noncoding RNA database (http://blast.ncbi.nlm.nih.gov/) to annotate rRNA, snRNA, and snoRNA. The remaining sequences were then searched against the mature miRNA of all animals in miRBase (Release 19.0) [Griffiths-Jones et al., 2008] to identify conserved miRNA homologs in common carp. Sequences which were identical or related (no more than one mismatch in the seed sequence and a few end nucleotides fluctuation in the entire length) to the reference mature miRNAs were annotated as miRNA candidates. The miRNA candidates were then clustered into categories according to sequence similarity, and sequences varying only in length and/or a few end nucleotides were gathered under the same miRNA identifier. Subsequently, all sequences that matched a miRNA in miRBase were searched against the zebrafish genome. The surrounding 300 bases flanking each small RNA sequence were obtained and their folding secondary structures were determined using the Mireap software (http://sourceforge.net/projects/mireap/) to determine if the sequences could form the characteristic hairpin secondary structure of pre-miRNA. If a hairpin structure with the free energy of hybridization lower than −20 kcal/mol was predicted, the RNA sequence was subjected to a MiPred analysis which predicts whether the input RNA sequence is a genuine pre-miRNA-like hairpin sequence [Jiang et al., 2007].

To identify potential novel miRNAs in common carp, all unannotated sequences were aligned against the zebrafish genome using SOAP [Li et al., 2008]. Sequences with perfect match or one mismatch were retained for further analysis. For each mapped sequence, hairpin folding was evaluated by sequence analysis to identify the presence of a stem loop with 18 or more base pairs, and the folding energy was calculated using the Mireap software (BGI-Shenzhen).

VALIDATION OF NOVEL COMMON CARP miRNAs USING RT-PCR ANALYSIS

To authenticate novel miRNAs, total RNA was extracted from the heart, liver, kidney, gill, gut, eye, spleen, and brain of 1-year-old common carp with TRIzol reagent (Takara). The RNAs from eight tissues were mixed together in equivalent concentrations, and then

treated with DNase I (RNase-free) (Takara). The total RNA was polyadenylated with ATP by *E. coli* poly(A) polymerase (Biolabs, New England). Then the poly(A) miRNAs were reverse transcribed with M-MLV Reverse Transcription Reagents (Invitrogen) and a poly (T) primer ligated with a RACE adapter. The forward primers used in the PCR that was run on an Applied Biosystems 7300 Sequence Detection system (Applied Biosystems, Foster City, CA), were specific to the miRNAs (Table S1). The PCR products were detected on 3% agarose gel. U6 small nuclear RNA was used as an endogenous control for miRNAs.

PREDICTION OF miRNA TARGETS, GO ENRICHMENT AND KEGG PATHWAY ANALYSIS

Because of the absence of whole and available genomic information for the common carp, the zebrafish genomic information was used to predict the potential target gene of miRNAs, the purpose of which is to obtain target genes as much as possible. This consideration is mainly based on that target sites in the 3′UTR of genes are highly conserved among closely related animals. The rules used for target prediction are based on those suggested by Schwab et al. [2005]. For the GO enrichment analysis [Harris et al., 2004], the Bonferroni Correction for the *P*-value was used to obtain a corrected *P*-value, and GO terms with corrected *P*-value ≤ 0.05 were defined as significantly enriched. For the KEGG pathway analysis [Kanehisa et al., 2008], genes with FDR ≤ 0.05 were considered as significantly enriched in target gene candidates.

RESULTS AND DISCUSSION

SOLEXA SEQUENCING OF SMALL RNAs

Through Solexa high throughput sequencing, 10,684,733 total reads were obtained; 89.51% of the reads were 21–23 nucleotides in length (Fig. 1). The length of the cleaned reads peaked at 22 nt (Fig. 1). After removal of the 5′ and 3′ adapters, pollution reads and reads smaller than 18 nucleotides, 10,603,456 high-quality clean reads were extracted, and 293,603 of the resulting unique small RNAs were annotated with the RFam 10.1 database. 475,829 reads of rRNA, tRNA, snoRNA, and other snRNAs were annotated and then removed from the following analysis (Fig. 2). The remaining 10,127,627 reads were retained for miRNA analysis. Given that no whole genome data for common carp are available, we aligned the selected small RNA sequences to the genome sequence of zebrafish (http://hgdownload.cse.ucsc.edu/downloads.html#zebrafish), which is the only completely sequenced genome of the cyprinidae fish family, to perform a distribution analysis using SOAP [Li et al., 2008]. The 8,942,168 reads that represent 40,572 unique sequences were perfectly mapped to the genome sequence of zebrafish (Fig. 3).

CONSERVED AND NOVEL miRNAs IN THE SPLEEN OF COMMON CARP

To identify conserved miRNAs in the spleen tissue of common carp, the clean sequences were annotated with the mature miRNA sequences of all animals deposited in miRBase (release 19.0). The 38,901 unique sequences (9,710,047 reads) with homologous to known miRNAs are annotated as conserved miRNAs, while the rest 204,758 unique sequences (417,580 reads) were unannotated (Fig. 2). To further validate these match miRNA sequences, a BLASTN search was performed against the zebrafish genome sequence. The genomic

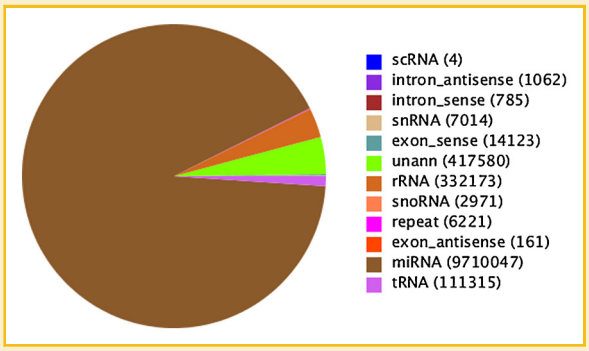

Fig. 2. Annotation of small RNAs derived from the spleen of the common carp. Clean reads were BLAST searched against the RFam 10.1 database to annotate rRNAs, tRNAs, snoRNAs, and other snRNAs.

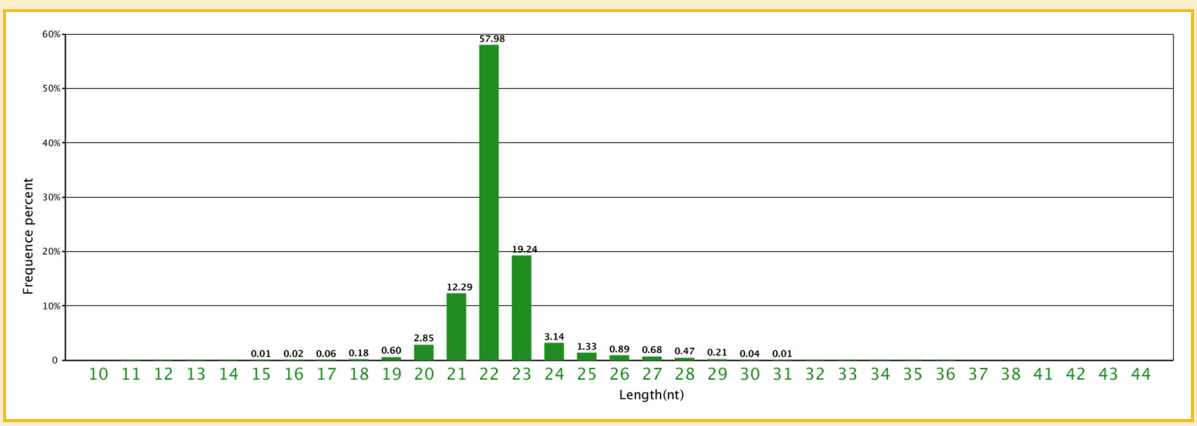

Fig. 1. Length distribution of small RNAs derived from the spleen of the common carp.

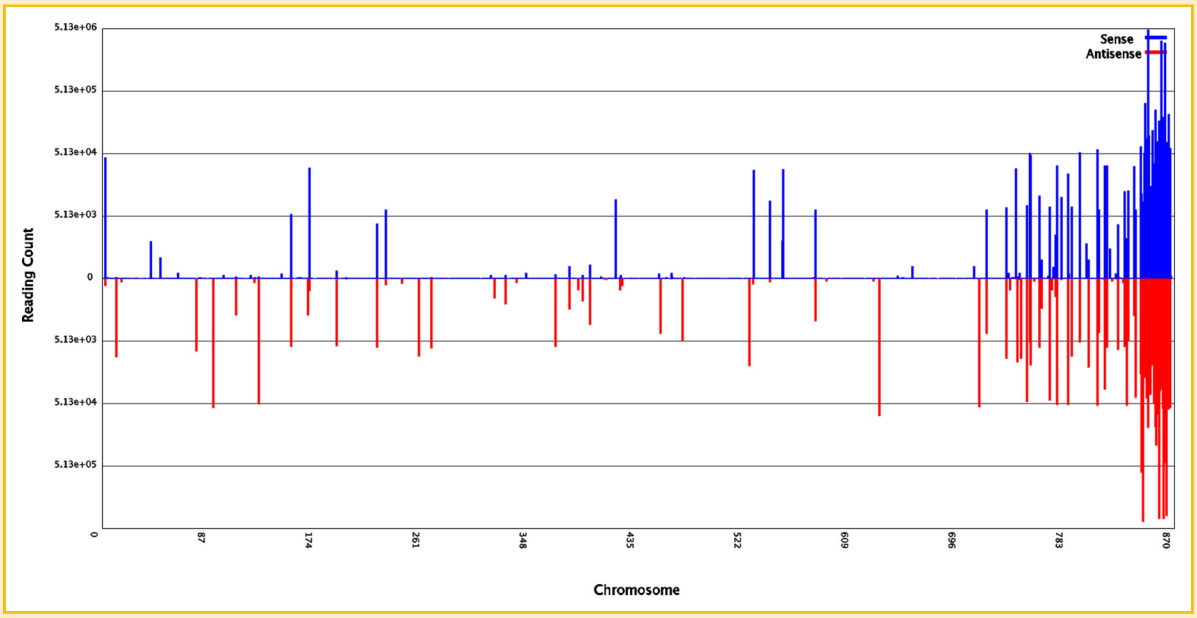

Fig. 3. Number and distribution of sequence reads mapped to the genome sequence of zebrafish.

sequences with a perfect match to the query sequences and their flanking regions were excised and tested for folding properties using the Mireap software (BGI-Shenzhen), requiring a free energy $\triangle G \leq$ -20 kcal/mol and the read sequence situated on one arm of the hairpin. The stem-loop structure was further interrogated using the program MiPred [Jiang et al., 2007]. Ultimum, the 269 genomic sequences encompassing the 194 conserved miRNAs were predicted to be capable of forming stable hairpin structures characteristic of miRNA precursors (Additional File S1 and Table S2). Of the 194 conserved miRNAs identified in this study, 123 miRNAs have been deposited in common carp in miRBase (20.0) (Table S2-1), 71 classified as new common carp miRNAs that have not been previously discovered in common carp but have been identified in other species (Table S2-2). All conserved common carp miRNAs were classified into known families or currently undefined groups on the basis of sequence similarity. Among the 194 conserved miRNAs, 97 miRNAs belong to 30 miRNA gene families, which were detected at the highly sequence reads in common carp spleen tissue (Table S3). Another 12 miRNAs are undefined groups, and the rest belongs to the respective miRNA all alone the gene families (data not shown).

The small RNAs sequences that did not match known miRNAs were compared with the zebrafish genome sequence to detect potential novel miRNAs. The flanking regions of sequence reads matching the zebrafish genome were subjected to secondary structure analysis using the Mireap software (BGI-Shenzhen). According to the criteria of potential miRNAs identity mentioned above, 14 short RNA producing loci corresponding to 12 unique sequence reads could be folded into step-loop structures (Additional File S2). This allowed us to identify 12 novel miRNAs in common carp (Table I).

CHARACTERIZATION OF miRNAs SEQUENCE IN THE SPLEEN OF COMMON CARP

The conserved miRNAs length ranges from 19 to 25 nt. Majority (58%, i.e., 113 out of 194) of the miRNAs are 22 nt in length, followed by 21 nt (19%), 23 nt (14%), 24 nt (3.9%), 20 nt (3.6%), 25 nt (1%), and 19 nt (0.5%). The mature miRNA lengths are similar to previous reports in other animal species [Li et al., 2010]. Among the 71 newly conserved miRNAs sequences identified in the spleen tissue of common carp, the 45 are perfectly (100%) matched, 22 have a difference of 1–2 and 4 have a difference of 3–4 nucleotides with the corresponding homologue miRNAs (Table S2-2). The results are the same as previously described by other groups, where the mature sequences have a difference of four nucleotides [Li et al., 2010]. In addition, the length of the novel miRNA sequences varied from 21 to 23 nt with a distribution peak at 21 nt, and their 5′ ends were most frequently uridine (U). Solexa sequencing results revealed the majority of the identified miRNAs have length and sequence heterogeneity in common carp spleen tissue. The length variations occurred predominantly in the 3′ end of miRNAs mainly in the form of trimming and/or as nucleotide addition variants (Additional File S1). These end-sequence variations are intriguing as they may allow miRNA variants to perform distinct roles by influencing miRNA/target mRNA hybrid duplex formation [Jazdzewski et al., 2009].

ABUNDANCE AND EXPRESSION OF miRNAs IN THE SPLEEN OF COMMON CARP

The sRNA sequencing allowed us to identify miRNAs and also to determine the expression levels of the miRNAs. The number of reads that could be aligned to each of the miRNAs was assumed to represent the expression level of the miRNA. We found a lot of divergence in the abundance of the different miRNAs, which varied

TABLE I. Comprehensive List of Novel MicroRNA Identified in the Spleen of Common Carp

miRNA*	Sequence (5′ → 3′)	Read count	% of reads	Position in the reference sequence
ccr-miR$_{new}$-1	GACTTGGTCTAAGCTCCTCAGT	58	0.00055	Zv9_scaffold3487:171746:171835:+
ccr-miR$_{new}$-2	TGCTGTCTCAGTCAGGTCCGGT	5	0.00005	chr10:9822228:9822297:−
ccr-miR$_{new}$-3	AGCACATGGTCTCTTACTTGTA	127	0.0012	chr10:36675708:36675786:−
ccr-miR$_{new}$-4	GATCGGGTGTCCGCGCTAAGT	9	0.00008	chr11:12532887:12532977:−
ccr-miR$_{new}$-5	TTACAATTAAAGGATATTTCTT	5	0.00005	chr12:13216983:13217063:−
				chr17:25795651:25795727:−
ccr-miR$_{new}$-6	TGGGGATATAAGGAAGTATGT	6	0.00006	chr17:5730418:5730495:+
ccr-miR$_{new}$-7	TTTAGCATGGTGGTGGAAAGA	6	0.00006	chr17:17720790:17720885:+
ccr-miR$_{new}$-8	TACATTCATTGATGTCGTTGGGT	73	0.00069	chr21:7721355:7721432:−
ccr-miR$_{new}$-9	CAGGACTTAACCCACTTGTGA	9	0.00008	chr3:13208962:13209043:+
				chr3:13304506:13304587:−
ccr-miR$_{new}$-10	TCTCTATTTGACTGTCAGCATG	11	0.0001	chr6:36906304:36906388:+
ccr-miR$_{new}$-11	ACAGCCCTGAAATTCAGACAG	7	0.00007	chr9:14238795:14238871:−
ccr-miR$_{new}$-12	TCAATTCCTGGGACCTACGCA	7	0.00007	chr9:30685753:30685848:−

Total number of reads representing each microRNA in the small RNA library obtained from the common carp spleen.
Percentage of total reads representing each microRNA in the small RNA library obtained from the common carp spleen.
*Complete list of microRNAs currently not present in the miRBase *C. carpio* miRNA database and not previously identified in any other species found in small RNA libraries of the common carp spleen.

from several counts for rare miRNAs to several million reads for the most abundant miRNAs (Table I and Table S4). In general, most of the novel miRNAs identified in common carp are relatively weakly expressed compared with conserved miRNAs. Except ccr-miR$_{new}$-1, ccr-miR$_{new}$-3, and ccr-miR$_{new}$-8, the absolute sequencing frequencies of these novel miRNAs was much lower in the spleen of common carp (Table I). The same phenomenon has also been observed for other species [Chi et al., 2011; Fu et al., 2011; Yan et al., 2012], which suggests that novel miRNAs are usually weakly expressed while conserved miRNAs are highly expressed.

Although these conserved miRNAs were sequenced at varying frequencies, some miRNAs such as ccr-let-7a, ccr-miR-21, ccr-miR-146a, and ccr-miR-462 dominated the miRNA library (Table II). These abundant miRNAs principally belong to 30 miRNA gene families, and their family members were at least or more than two expressed in the spleen of common carp (Table S3). Generally speaking, abundant miRNAs play fundamental and broad regulatory functions in maintaining biological processes. The most abundant miRNA in the spleen of common carp was ccr-let-7a, with almost four million reads corresponding to 37.61% of all clean reads (Table II). Let-7 is known to be one of the most conserved miRNA family in animal. Nine members of the let-7 miRNA family including let-7a, let-7b, let-7c, let-7d, let-7f, let-7g, let-7i, let-7j, and let-7k were characterized in the spleen of common carp by high throughput sequencing, all of which have a high read frequency (ranging from 6,957 to 3,988,125). Previous studies in mammals have also indicated that the let-7 gene family can regulate the expression of major cytokine inducible proteins in response to microbial challenge [Mondol and Pasquinelli, 2012; Wang et al., 2012]. Possibly because of vital roles in common carp, let-7 was identified as the most abundant miRNA family. Moreover, the second most abundant miRNA was ccr-miR-21, with 8.45% of all clean reads (Table II). In agreement with this result, a study by Chi et al. [2011] also observed that miR-21 is highly expressed in the two carps: 178,007 sequences in bighead carp and 270,728 sequences in silver carp, by high throughput sequencing for the small RNA library derived from five organs (heart, liver, brain, spleen, and kidney). These results suggested that miR-21 plays a crucial role in immunological processes in fish. In contrast, the sequencing frequency of miR-135c, miR-153c, miR-454a, miR-457a, miR-729, miR-730, miR-734, miR-4837, and so on was extremely low in common carp spleen library (Table S4). It is possible that these miRNAs are expressed at very low levels, in limited cell types, and/or under limited circumstances.

Several recent reports have described miRNAs in common carp from the skeletal muscle tissue [Yan et al., 2012] and the pooled tissue sample including brain, skin, liver, muscle, spleen, head kidney, body kidney, intestine, gill and heart [Zhu et al., 2012]. The comparison was performed between the miRNAs discovered in the common carp spleen tissue with those identified in the skeletal muscle [Yan et al., 2012] and the pooled tissue sample [Zhu et al., 2012]. The result shows that most of miRNAs identified in this study are discovered in the skeletal muscle and/or pooled tissue sample, but the miRNAs including miR-1388, miR-181d, miR-2184, miR-2187, miR-2188, miR-29c, miR-458b, miR-737, miR-3120, miR-4837, miR-5109, miR-6651, miR-696, miR-7132, and miR-7133 were expressed only in spleen tissue of common carp (Table S5). This result indicates that these miRNAs showed specificity expression in the spleen.

CONSERVATION ANALYSIS OF miRNAs IN THE SPLEEN OF COMMON CARP

Many miRNAs were highly conserved among organisms. Conserved miRNAs likely played an important role in regulating basic cellular and developmental pathways from lower to higher organisms [Glazov et al., 2008]. The conservation analysis of miRNAs may help us infer the functions of miRNAs in the spleen of common carp based on their known functions in other species. Therefore, we randomly selected typical model animals to analyze the conservation of the known miRNA identified in spleen of common carp in vertebrates and invertebrates. The result of family analysis showed

TABLE II. Most Frequently Sequenced *C. carpio* miRNAs in the Spleen Small RNA Library

miRNA	Reads	% of reads
ccr-let-7a	3,988,125	37.61
ccr-miR-21	896,416	8.45
ccr-let-7f	668,659	6.31
ccr-miR-146a	286,495	2.70
ccr-miR-462	281,270	2.65
ccr-let-7g	237,487	2.24
ccr-miR-22a-3p	209,190	1.97
ccr-let-7k	203,698	1.92
ccr-let-7b	169,513	1.60
ccr-let-7d	129941	1.23
ccr-let-7c	121,108	1.14
ccr-miR-101a	102,334	0.97
ccr-miR-143	91,618	0.86
ccr-miR-128	82,669	0.78
ccr-miR-140-3p	73,876	0.70
ccr-miR-146b	54,849	0.52
ccr-miR-107	52,506	0.50
ccr-miR-10c	50,358	0.47
ccr-miR-122	49,702	0.47
ccr-miR-142-5p	33,695	0.32
ccr-miR-199-3p	30022	0.28
ccr-miR-26a	29,268	0.28
ccr-miR-25	28784	0.27
ccr-miR-181a-5p	23,597	0.22
ccr-miR-144-5p	22,584	0.21
ccr-miR-29a	22431	0.21
ccr-miR-221-3p	21127	0.20
ccr-miR-30d	20744	0.20
ccr-miR-145-5p	16,882	0.16
ccr-let-7i	16,592	0.16
ccr-miR-2188-5p	15,465	0.15
ccr-miR-92a	12,343	0.12
ccr-miR-148	12,079	0.11
ccr-miR-150	11,957	0.11
ccr-miR-210-5p	11,073	0.10
ccr-miR-181c	11,009	0.10
ccr-miR-181b	11,004	0.10
ccr-miR-222-3p	10,795	0.10
ccr-miR-217	10,568	0.10
ccr-miR-23a	10050	0.09

Percentage of total reads representing each microRNA in the small RNA library obtained from the common carp spleen.

that 74 conserved miRNA families were clustered into three groups based on their phylogenetic distributions (Fig. 4 and Fig. S1). Twenty-three miRNA families were shared by both protostomes and deuterostomes; 46 miRNA families were present only in vertebrates; and the remaining five miRNA families (mir-430, mir-722, mir-724, mir-734, and mir-738) were identified only in fish and were possibly fish-specific miRNAs. Meanwhile, to analyze the conservation of common carp miRNAs, we compared them to those identified in model animals, including worm (*Caenorhabditis elegans*), fly (*Drosophila melanogaster*), frog (*Xenopus tropocalis*), zebrafish (*Danio rerio*), chicken (*Gallus gallus*), mouse (*Mus musculus*), and human (*Homo sapiens*). Sequence similarity searches showed that most of the miRNAs were conserved across many species. Among them, only let-7a is highly conserved from worm to human, eight common carp miRNAs are conserved both in vertebrates and invertebrates, 107 miRNAs are conserved only in vertebrate model animals (Table S6), although there exist base edition and length change of sequences across the different animal species.

Through literature mining, we found that 26 miRNAs are known to be involved in animal immunity, and these miRNAs were also found in the spleen of common carp (Table III). Among the 26 miRNAs, except for miR-21, miR-146a, miR-146b, miR-150, and miR-15b, the other miRNAs were highly conserved in multiple animal species (Table S6). Especially, four members of miR-181 gene family including miR-181a, miR-181b, miR-181c, and miR-181d were sequenced at high frequency (ranging from 11,009 to 23,597) in the spleen of common carp. Studies on these miRNAs in mammals indicate that miR-181 family is involved in multiple roles in immune regulation and disease. An earlier report found that increasing miR-181a expression in mature T cells augments their sensitivity to peptide antigens, while inhibiting miR-181a expression in immature T cells reduces sensitivity and impairs both positive and negative selection [Li et al., 2007]. A study by Feng et al. [2012] confirmed that miR-181a in spleen CD4+ T lymphocytes play proinflammatory roles in a murine model of asthma. Another study by Cichocki et al. [2011] demonstrated that miR-181 promotes human NK cell development by regulating Notch signaling. In addition, miR-122 is involved in the responses to bacterial infection [Wu et al., 2012], and has the higher expression in the common carp spleen (49,702 sequences: 0.47%) (Table II). But, its expression abundance was markedly lower than the expression level of bighead carp (1,808,356 sequences: 48%) and silver carp (556,153 sequences: 15%) in the pooled sample from five organs (heart, liver, brain, spleen, and kidney) [Chi et al., 2011]. Therefore, these results indicate that the correlation between miRNA sequence conservation and expression conservation is weak. Otherwise, miR-155 plays important role in normal immune function including the macrophage inflammatory response [O'Connell et al., 2007], the generation of immunoglobulin [Vigorito et al., 2007], and so on. miR-150, a microRNA expressed in mature B and T cells, controls B cell differentiation by targeting the transcription factor c-Myb [Xiao et al., 2007]. Nevertheless, miR-150 blocks early B cell development when expressed prematurely [Zhou et al., 2007]. In a word, these previous observations are very in agreement with the expression pattern corresponding miRNAs in our sequencing result, suggests that these conserved miRNAs may play roles similar to those of their orthologs in regulating development and function of common carp spleen.

Recently, a number of lineage-specific miRNAs and species-specific miRNAs were discovered [Berezikov et al., 2006]. In our study, we have also discovered some miRNAs that only conserved in fish species (Table IV). Moreover, three miRNAs (ccr-miR-7133, ccr-miR-7132, and ccr-miR-551) were only identified in common carp. The result indicates these miRNAs were fish-specific and might play a key role in the evolutionary process of fish.

VALIDATION OF NOVEL COMMON CARP miRNAs

Since whole genome data for the common carp are not available, the prediction of novel miRNAs was based on the zebrafish genome in this study. To validate the reliability of the identified miRNAs, their expression in the RNAs from the pooled tissue was examined by RT-PCR. The 12 novel potential miRNAs and four new conserved

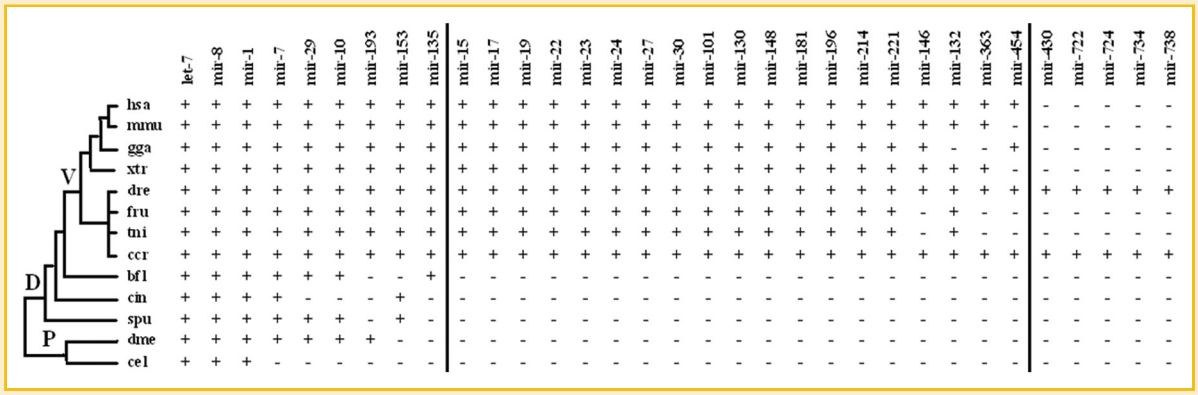

Fig. 4. Conserved miRNAs in the spleen of common carp. The presence of miRNA is indicated by plus (+); the absence of miRNA is indicated by minus (−). Abbreviations: hsa, *Homo sapiens*; mmu, *Mus musculus*; gga, *Gallus gallus*; xtr, *Xenopus tropicalis*; dre, *Danio rerio*; fru, *Takifugu rubripes*; tni, *Tetraodon nigroviridis*; ccr, *Cyprinus carpio*; bfl, *Branchiostoma florida*; cin, *Ciona intestinalis*; spu, *Strongylocentrotus purpuratus*; dme, *Drosophila melanogaster*; cel, *Caenorhabdits elegans*; P, protostomia; D, deutostomia; V, vertebrata.

TABLE III. The miRNAs Both Identified in the Spleen of Common Carp and Demonstrated to Be Immunity-Related in Other Species

miRNA	Frequency	Function	Refs.
miR-21	896,416	LPS-induced innate immune response	Moschos et al. [2007]
miR-146a	286,495	Innate immune response	Taganov et al. [2006]
		Negative regulator of LPS signaling	Dai et al. [2008]
		Proinflammatory	Feng et al. [2012]
miR-146b	54,849	Proinflammatory	Feng et al. [2012]
miR-101a	102,334	Acute inflammatory immune responses	Xia et al. [2011]
miR-140	73,876	LPS-induced innate immune response	Moschos et al. [2007]
miR-122	49,702	Responses to bacterial infection	Wu et al. [2012]
miR-142	33,695	Highly expressed in hematopoietic tissues	Merkerova et al. [2008]
miR-25	28,784	LPS-induced innate immune response	Moschos et al. [2007]
miR-144	22,584	Erythropoiesis	Dore et al. [2008]
miR-221	21,127	Erythropoiesis and erythroleukemic cell growth	Felli et al. [2005]
let-7i	16,592	Against *Cryptosporidium parvum* infection	Chen et al. [2007]
miR-150	11,957	Highly expressed in B- and T-lymphocytes	Merkerova et al. [2008]
		B and T cell development	Zhou et al. [2007]
		B cell differentiation	Xiao et al. [2007]
miR-181a	23,597	T cell sensitivity to antigens	Li et al. [2007]
		Proinflammatory	Feng et al. [2012]
miR-181c	11,009	LPS-induced innate immune response	Moschos et al. [2007]
		B and T cell development	Chen et al. [2004]
miR-222	10,795	Acute inflammatory immune responses induced	Xia et al. [2011]
		Erythropoiesis and erythroleukemic cell growth	Felli et al. [2005]
miR-192	8833	Acute inflammatory immune responses induced	Xia et al. [2011]
		Responses to bacterial infection	Wu et al. [2012]
miR-125b	6,866	LPS signaling	Tili et al. [2007]
miR-100	6,029	LPS-induced innate immune response	Moschos et al. [2007]
miR-30c	2,774	Differential expression in peripheral blood cells	Merkerova et al. [2008]
miR-15b	2,238	Chronic lymphocytic leukemia	Calin et al. [2002]
miR-27b	613	LPS-induced innate immune response	Moschos et al. [2007]
		Acute inflammatory immune responses induced	Xia et al. [2011]
miR-24	599	Differential expression in peripheral blood cells	Merkerova et al. [2008]
miR-155	405	T helper cell differentiation	Thai et al. [2007]
		LPS signaling	Tili et al. [2007]
		Erythropoiesis	Masaki et al. [2007]
		Macrophage inflammatory response	O'Connell et al. [2007]
		Requirement for normal immune function	Rodriguez et al. [2007]
		Immunoglobulin class-switched plasma cells	Vigorito et al. [2007]
		Antiviral response	Dang et al. [2008]
miR-187	208	LPS-induced innate immune response	Moschos et al. [2007]
miR-194	41	LPS-induced innate immune response	Moschos et al. [2007]
		Responses to bacterial infection	Wu et al. [2012]
miR-124c	28	Acute inflammatory immune responses induced	Xia et al. [2011]

TABLE IV. Common Carp miRNAs Only Conserved in the Teleosts

miRNA	Frequency	Conserved
ccr-miR-10d	77	dre fru hhi ipu ola tni
ccr-miR-22b	605	dre fru ipu tni
ccr-miR-153b	21	dre fru ipu tni
ccr-miR-125c	175	dre ipu ola
ccr-miR-737	186	dre hhi ipu
ccr-miR-723	11	dre hhi
ccr-miR-462	281,270	dre ipu ola
ccr-miR-135c	7	dre ipu
ccr-miR-457a	6	dre ipu
ccr-miR-724	879	dre ipu
ccr-miR-730	5	dre ipu
ccr-miR-2187	304	dre ipu
ccr-miR-18c	306	dre
ccr-miR-19d	169	dre ola
ccr-miR-729	9	dre ola
ccr-miR-722	230	dre
ccr-miR-726	258	dre
ccr-miR-734	7	dre
ccr-miR-153c	9	dre

Abbreviations: dre, *Danio rerio*; fru, *Fugu rubripes*; hhi, *Hippoglossus hippoglossus*; ipu, *Ictalurus punctatus*; ola, *Oryzias latipes*; tni, *Tetraodon nigroviridis*.

miRNAs were selected. The PCR results showed that all the selected miRNAs were expressed in mixed tissue of heart, liver, kidney, gill, gut, eye, spleen, and brain (Fig. 5), indicating that these miRNAs were correctly identified and truly expressed in the common carp.

MicroRNA TARGET PREDICTIONS, GO ENRICHMENT, AND KEGG PATHWAY ANALYSIS

The identification and analysis of miRNA targets is an important step to better understand the molecule functions of miRNAs. Based on sequence complementarity between miRNAs and their mRNA targets, potential target sequences for the 56 highly conserved miRNAs between common carp and zebrafish (Table S6) were identified by searching for antisense hits in the reference RNA sequences of the zebrafish. A total of 9,212 targets were predicted for the 56 highly conserved miRNAs (data not shown).

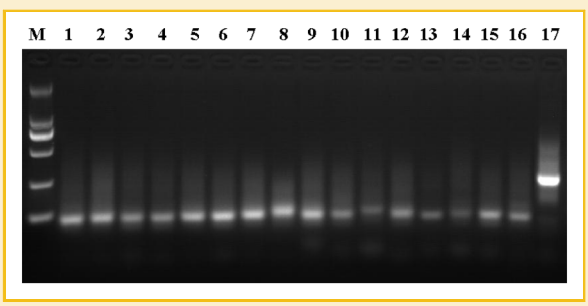

Fig. 5. The expression of novel miRNAs in pooled total RNAs from eight common carp tissues including heart, liver, kidney, gill, gut, eye, spleen, and brain were detected by RT-PCR. The product were run on a 3% agarose gel. Lanes from 1 to 17 indicate miR-146b, miR-150, miR-30c, miR-462, miR_{new}-1, miR_{new}-2, miR_{new}-3, miR_{new}-4, miR_{new}-5, miR_{new}-6, miR_{new}-7, miR_{new}-8, miR_{new}-9, miR_{new}-10, miR_{new}-11, miR_{new}-12, and U6 snRNA, respectively. M: DL2 000 DNA Marker.

Subsequently, GO enrichment analysis and KEGG pathway analysis for these predicted target genes were performed to identify the functions and pathways that are actively regulated by miRNA in spleen of common carp. The results of GO enrichment analysis showed that these potential targets are associated with a broad range of biological functions, for instance, immune regulation, transcriptional regulation, and metabolism. Of these, GO terms with *P*-value as good or better than one, which are involved in immune system process, defense response, and response to stimulus, were listed in the Table S7. These terms are broadly involved in proper immune system development and a variety of other aspects of the immune system (Table S7). Especially, the functional categories of GO terms associated with immune system development, hematopoietic or lymphoid organ development, lymphocyte activation, immune response, response to external stimulus, and so on, were significantly overrepresented among the predicted target genes (Table V).

The results of pathway analysis indicate that the predicted target genes were significantly enriched in a broad range of pathways including the immune/defense relevant pathways (Table VI), pathogen infection (Table S8-1), autoimmune disease (Table S8-2), apoptosis, endocytosis, cell cycle, cell adhesion molecules, focal adhesion, cell junction (Table S8-3), MAPK signaling pathways (Table S8-4), and metabolism (Table S8-5). Intriguingly, the immune/defense relevant pathways included in the various aspects of B cell receptor signaling pathway, T cell receptor signaling pathway, regulation of autophagy, phagosome, Fc gamma R-mediated phagocytosis, chemokine signaling pathway, Toll-like receptor (TLR) signaling pathway, and so on (Table VI), which suggested that the conserved miRNAs perhaps participate in immune/defense regulation of the common carp by these pathways. Among them, TLRs is known to be recognize conserved microbial products and defend against pathogenic attack by initiating an immune response via signaling pathways in the host, and it is strongly associated with both the innate immune and adaptive immune systems in fish [Bilodeau-Bourgeois et al., 2008]. Several miRNAs have been shown to control TLR signaling in many immune and inflammatory pathways. For instance, miR-146a inhibits oxidized low-density lipoprotein-induced lipid accumulation and inflammatory response via targeting TLR 4 [Yang et al., 2011], miR-21 inhibits TLR 2 agonist-induced lung inflammation in mice [Case et al., 2011], miR-26a negatively regulates TLR 3 expression of rat macrophages and ameliorates pristane induced arthritis in rats [Jiang et al., 2014], miR-92a negatively regulates TLR-triggered inflammatory response in macrophages by targeting MKK4 kinase [Lai et al., 2013], let-7i regulates TLR 4 expression and contributes to against Cryptosporidium parvum infection [Chen et al., 2007]. Moreover, B-cell receptor (BCR) is essential for normal B-cell development and maturation, and apoptosis induced by BCR signaling is critical for antigen-driven selection. An earlier report identify that BCR activation induces BIC/miR-155 expression through a conserved AP-1 element [Yin et al., 2008]. A study by Kluive and Chen [2012] demonstrate that microRNAs, such as miR-150, miR-181a1b1 and miR-17–92, regulate BCR signaling-induced apoptosis, and may have important roles in the regulation of B cell-

TABLE V. Significant Enrichment GO Term Involved in Immunity and Response to Stimulus

Gene ontology term	Cluster frequency (target genes)	Corrected *P*-value
Response to stimulus	32.8% (3,022)	1
Response to external stimulus	7.7% (708)	1
Regulation of response to stimulus	7.5% (688)	1
Immune system process	6.3% (582)	1
Immune system development	2.7% (248)	1
Regulation of immune system process	2.6% (240)	1
Hematopoietic or lymphoid organ development	2.5% (233)	1
Response to osmotic stress	1.8% (169)	1
Hemopoiesis	1.7% (159)	1
Response to endogenous stimulus	1.4% (130)	1
Defense response	1.4% (125)	1
Regulation of immune response	1.1% (103)	1
Leukocyte activation	1.1% (97)	1
Lymphocyte activation	0.9% (85)	1
Activation of immune response	0.9% (84)	1
Immune response-activating signal transduction	0.9% (80)	1
Immune response-regulating signaling pathway	0.9% (80)	1
Regulation of cytokine production	0.8% (74)	1
Leukocyte differentiation	0.8% (71)	1
Response to bacterium	0.6% (59)	1
Immune response	0.6% (57)	1
Response to molecule of bacterial origin	0.6% (52)	1
Lymphocyte differentiation	0.5% (49)	1
Regulation of innate immune response	0.5% (48)	1
Positive regulation of innate immune response	0.5% (48)	1
Response to cytokine stimulus	0.5% (48)	1
Innate immune response-activating signal transduction	0.5% (48)	1
Activation of innate immune response	0.5% (48)	1
Toll-like receptor signaling pathway	0.5% (47)	1
T cell activation	0.5% (45)	1
Immune response-regulating cell surface receptor signaling pathway	0.5% (44)	1
Immune response-activating cell surface receptor signaling pathway	0.5% (44)	1
Regulation of endocytosis	0.4% (36)	1
Antigen processing and presentation	0.4% (36)	1
T cell differentiation	0.3% (30)	1
Cytokine production	0.3% (29)	1
Regulation of lymphocyte differentiation	0.3% (28)	1
Leukocyte proliferation	0.3% (24)	1
Adaptive immune response	0.2% (20)	1
Production of molecular mediator of immune response	0.2% (18)	1
Regulation of B cell activation	0.2% (17)	1
T cell proliferation	0.2% (17)	1
Immunoglobulin production	0.2% (14)	1

mediated tolerance and immunity. Noteworthy, these miRNAs in the previous research above were also identified in the common carp spleen. Collectively, these pathway and biological process analyses were consistent with our Solexa sequencing results, suggesting that the conserved miRNAs may be closely related to the immune regulation of common carp.

TABLE VI. Significant Enrichment Pathways Involved in the Immune and Defense

Pathway	Target genes with pathway annotation (frequency)	*P*-value
Phagosome	296 (2.54%)	1
Chemokine signaling pathway	211 (1.81%)	1
Leukocyte transendothelial migration	210 (1.8%)	1
Lysosome	163 (1.4%)	1
T cell receptor signaling pathway	147 (1.26%)	1
Fc gamma R-mediated phagocytosis	135 (1.16%)	1
Antigen processing and presentation	130 (1.12%)	1
Complement and coagulation cascades	122 (1.05%)	1
Natural killer cell mediated cytotoxicity	121 (1.04%)	1
Toll-like receptor signaling pathway	104 (0.89%)	1
B cell receptor signaling pathway	93 (0.8%)	1
Fc epsilon RI signaling pathway	90 (0.77%)	1
NOD-like receptor signaling pathway	90 (0.77%)	1
Hematopoietic cell lineage	70 (0.6%)	1
Intestinal immune network for IgA production	66 (0.57%)	1
RIG-I-like receptor signaling pathway	60 (0.51%)	1
Regulation of autophagy	24 (0.21%)	1

CONCLUSIONS

In this study, we used Solexa sequencing to identify 194 distinct conserved miRNAs, consisting of 123 previously reported miRNAs and 71 new miRNAs, from the spleen tissue of common carp. The expression levels of these conserved miRNAs displayed a large range, and those abundantly expressed could be classified into 30 miRNA families. Meanwhile, we validated the 12 novel miRNAs by RT-PCR analysis, which confirmed that these miRNAs were truly expressed in the common carp. Furthermore, we also analyzed potential functions of miRNAs in the spleen of common carp by GO enrichment analysis and KEGG pathway analysis of the predicted target gene. Our results for the first time provides data for the identification and characterization of miRNAs in the spleen of common carp. The identification and characterization of miRNAs involved in immune have facilitated the study of common carp miRNA function during the disease processes, which help in better understanding of the mechanisms underlying disease resistance/susceptibility in fish.

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