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Comprehensive identification and profiling of host miRNAs in response to Singapore grouper iridovirus (SGIV) infection in grouper (*Epinephelus coioides*)

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ABSTRACT

microRNAs (miRNAs) are an evolutionarily conserved class of non-coding RNA molecules that participate in various biological processes. Employment of high-throughput screening strategies greatly prompts the investigation and profiling of miRNAs in diverse species. In recent years, grouper (*Epinephelus* spp.) aquaculture was severely affected by iridoviral diseases. However, knowledge regarding the host immune responses to viral infection, especially the miRNA-mediated immune regulatory roles, is rather limited. In this study, by employing Solexa deep sequencing approach, we identified 116 grouper miRNAs from grouper spleen-derived cells (GS). As expected, these miRNAs shared high sequence similarity with miRNAs identified in zebrafish (*Danio rerio*), pufferfish (*Fugu rubripes*), and other higher vertebrates. In the process of Singapore grouper iridovirus (SGIV) infection, 45 and 43 miRNAs with altered expression (>1.5-fold) were identified by miRNA microarray assays in grouper spleen tissues and GS cells, respectively. Furthermore, target prediction revealed 189 putative targets of these grouper miRNAs.

1. Introduction

microRNAs (miRNAs) are endogenous small noncoding RNAs (ncRNAs) with 22–25 nucleotides (nt) in length, which are widely expressed by diverse eukaryotic organisms and viruses (Cai et al., 2006; Du and Zamore, 2005). Canonical miRNA biosynthesis initiates in nucleus, where miRNA genes are typically transcribed by RNA polymerase II to generate primary transcripts (pri-miRNA) (Lee et al., 2004). After that, the hairpin portion that exists in pri-miRNA is recognized and cleaved by RNase III endoribonuclease Drosha/DGCR8 to yield a 60–70 nt precursor miRNA (pre-miRNA) which is then transported to cytoplasm by exportin-5 in a Ran-GTP-dependent manner (Lee et al., 2004). In the cytoplasm, the pre-miRNA is further processed by another RNase III endoribonuclease-Dicer, and produces an imperfect short RNA duplex intermediate (Bernstein et al., 2001). Finally, the mature miRNA duplex is assembled into the miRNA-induced silencing complex (miRISC) where the seed sequence of the mature miRNA strand (positions 2–8 from the 5′ end) acts as a guide for individual miRISC to specifically recognize the 3′UTR of target mRNA, and thus promotes mRNA degradation and/or repress mRNA translation (Meister and Tuschl, 2004).

It is now well known that this class of evolutionally conserved ncRNAs play essential roles in diverse biological processes, such as cell proliferation, differentiation, apoptosis, oncogenesis and organ development (Bartel, 2004; Bushati and Cohen, 2007; Lee et al., 1993; Lu et al., 2005; Stefani and Slack, 2008). In the past decade, the roles of miRNAs in regulation of immune and inflammatory responses have also been extensively appreciated (Chen et al., 2013; Taganov et al., 2007; Xiao and Rajewsky, 2009). Investigations on miRNAs of immune relevance are indispensable for understanding the host–pathogen interactions, and these miRNAs may represent a new set of therapeutic targets for immune-related disease control (Iorio and Croce, 2012; Rederstorff and Huttenhofer, 2010).

The high-throughput screening approaches, such as Solexa deep sequencing and miRNA microarray assays, have emerged as powerful tools for miRNA profiling and novel miRNAs identification (Wang et al., 2008; Zhang et al., 2014). Given the evolutionarily conserved characteristic of miRNAs, these high-throughput screening strategies provide unprecedented insights into the miRNAs dynamics in different physiological or physiopathological conditions,

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especially for those species with limited genomic information. For example, by applying Solexa deep sequencing technology, Huang and colleagues identified 63 miRNAs from shrimp-*Marsupenaeus japonicus* and found that 31 of them were differentially expressed after white spot syndrome virus (WSSV) infection (Huang et al., 2012). Similar studies were performed in *Spiroplasma eriocheiris* challenged crayfish (Ou et al., 2013).

Grouper (*Epinephelus* spp.) are widely cultured marine food fish species in China and Southeast Asian countries. In recent years, accompanying the fast development of intensive large-scale aquaculture, various diseases, especially viral diseases have posed severe threat to grouper aquaculture production. Notably, Singapore grouper iridovirus (SGIV) is one of the most important viral pathogens which can cause >90% mortality in cultured grouper (Qin et al., 2003). However, due to the limited availability of grouper genome information, the molecular mechanisms of SGIV pathogenesis and virus–host interactions are yet largely unknown, and thus prevent the development of effective anti-viral strategies. In our previous studies, we have employed suppression subtractive hybridization (SSH) and cDNA microarray, as well as Roche's 454 pyrosequencing platform to investigate differentially expressed genes in immune organs of grouper after SGIV infection (Huang et al., 2011; Xu et al., 2010). These studies examined transcriptome-wide gene expression profiles during the *in vivo* SGIV infection, and provide extensive information on the host responses to viral infection. Considering the important roles of miRNAs in host–virus interactions, a comprehensive characterization of host miRNAs related to SGIV infection is indispensable for a deep understanding of the virus–cell interactions and pathogenesis.

In this study, we first identified 116 miRNAs expressed in grouper spleen cells by Solexa deep sequencing. During *in vivo* and *in vitro* viral infection, the expression profiles of these miRNAs were examined by miRNA microarray assays. Meanwhile, we performed multiple *in silico* analysis and identified a number of potential targets of these miRNAs. These data will help to uncover the complexity of immune regulatory networks mediated by grouper miRNAs during SGIV infection.

2. Materials and methods

2.1. Cells, virus, fish and sample preparation

Grouper spleen (GS) cells (Huang et al., 2009) were grown in Leibovitz's L-15 medium that contained 10% FBS at 25 °C. SGIV (strain A3/12/98) was originally isolated from diseased brown-spotted grouper (*Epinephelus tauvina*), and the infection and propagation of SGIV in cell cultures was performed as described previously (Qin et al., 2003). Fifteen-centimeter-long healthy groupers (*E. coioides*) were obtained from a local fish farm in Guangzhou, Guangdong Province of PR China. After maintained in a recirculating seawater system at 25–30 °C for 2 weeks, groupers were challenged by intraperitoneal injection with 0.2 ml of the SGIV suspension (1×10^5 TCID50/ml). Meanwhile, an equal volume of PBS was injected as a control. At 48 h p.i., 6 fish were randomly selected from two groups for spleen tissue sampling, respectively. Small RNAs was isolated from spleens using TRIzol reagent (Life Technologies, USA) according to manufacturer's protocol. Then equal amounts of small RNAs from spleen tissues of 6 fish in each group were pooled and used for microarray assay.

2.2. Solexa deep sequencing of small RNAs

RNA isolation and Solexa sequencing were performed as described previously (Yan et al., 2011). Briefly, normal GS cells cultured in 25-cm^2 flasks were collected for total RNA extraction using TRIzol reagent (Life Technologies, USA). The RNA was digested with DNase I (Life Technologies, USA) to eliminate DNA contamination. The quality and integrity of the total RNA was evaluated by electrophoresis and Agilent 2100 BioAnalyzer (Agilent Technologies, USA). After electrophoresing on 15% polyacrylamide–8 M urea gel, the small RNAs with about 30 nt were extracted, and a pair of Solexa proprietary adaptors as PCR primer was ligated to both 5′ and 3′ ends of the RNAs. After reverse transcription reaction from the RNAs, the resulting cDNA was amplified to produce sequencing libraries. The cDNA library was sequenced using the Illumina Genome Analyzer (Illumina, USA). Then the sequences reported in this paper were deposited in the Gene Expression Omnibus (GEO Accession number GSE64445).

2.3. In silico *analysis of small RNA reads*

After eliminating low quality reads, vector and adaptor sequences and redundancy contaminants, the processed data with size ≥18 nt and ≤26 nt length were used for miRNA prediction. Since grouper whole-genome sequence is unknown, the data were aligned with the zebrafish (*Danio rerio*) genome using SOAP v1.11 (Short Oligonucleotide Alignment Program) (http://soap.genomics.org.cn) (Li et al., 2008). Sequences that matched to rRNA, tRNA, snRNA and snoRNA in Rfam were discarded. Short RNA sequences originated from highly repeated elements were annotated as repeat-associated RNAs and also discarded. To identify conserved miRNAs between grouper and zebrafish, the unique small RNA sequences were analyzed by BLAST search against zebrafish miRNAs in miRBase v20.0 (http://www.mirbase.org/), and sequences with perfect match were considered as conserved miRNAs between two fish species. Subsequently, the remaining sequences were mapped to grouper EST database at NCBI (3548 ESTs). A total of 100 nt of DNA sequence flanking each side of the mapped ESTs was selected to predict hairpin RNA structures using Mfold software and analyzed by MIREAP (http://sourceforge.net/projects/mireap/) (Zuker, 2003).

2.4. miRNA microarray assay

Total RNA containing small RNAs was isolated using the *mir*Vana™ miRNA Isolation Kit (Ambion, USA). Then the quantity of the total RNA was accessed by NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, USA), and the integrity of the RNA was measured using the Agilent 2100 Bioanalyzer (Agilent Technologies, USA). Only A260/A280 ratio lies between 1.8 and 2.0 and RNA integrity number more than 7.0 can be accepted. After evaluating the quality and integrity of total RNA, miRNA microarray profiling was performed using Affymetrix®. GeneChip® miRNA 3.0 Array (Affymetrix, USA) contains all known zebrafish miRNAs (248 unique mature sequences). In brief, 1 μg of total RNA was ligated with poly-A tail and labeled with biotinylated DNA signal using FlashTag Biotin HSR RNA Labeling Kit (Affymetrix, USA). Then the labeled samples were hybridized to the arrays on Hybridization Oven 640 (Affymetrix, USA) at 48 °C and 60 rpm for 16 hours. After washing and staining on Fluidics Station 450 (Affymetrix, USA), the array was finally scanned on a GeneChip Scanner 3000 (Affymetrix, USA). miRNA QC Tool was used for data summarization, normalization, and quality control. After that, Affymetrix GeneChip Command Console software (version 4.0, Affymetrix) was used to analyze array images to get raw data and then offered RMA normalization. Next, Genespring software (version 12.5, Agilent Technologies, USA) was used to proceed the following data analysis. miRNAs with flags in "P (present)" were chosen for further data analysis. Differentially expressed miRNAs were then identified by fold change, and the threshold set for up- and down-regulated genes was a fold change ≥1.5.