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Short communication

Transcriptome comparative analysis revealed poly(I:C) activated RIG-I/MDA5-mediated signaling pathway in miiuy croaker

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ABSTRACT

Miiuy croker (*Miichthys miiuy*) as an important economical aquaculture species has challenged many more diseases caused by various pathogens recently. To better explore the immune response to virus, we have analyzed the transcriptome profiling of miiuy croaker challenged with poly(I:C) synthetic analog of virus dsRNA. We have obtained differentially expressed genes (DEGs) with up/down-relevant from comparison of the Ctrl and Poly transcriptome libraries. Through GO and KEGG enrichment analysis, immune-relevant DEGs whose expression are significantly rise or fall after challenged have been identified and classified. In order to detailedly analysis host immune response patterns for dsRNA virus, we have performed a map based on RIG-I/MDA5-mediated and TLR3-mediated signaling pathway which both induced type I IFNs response. In this pathway, both MDA5 and LGP2 are important RLRs in host surveillance against infection of dsRNA viruses and induce type I IFNs response which subsequently form a transcription factor complex ISGF3 that promote downstream genes referred to as ISGs to inhibits virus replication.

1. Introduction

Miiuy croaker, *Miichthys miiuy*, due to its taste, nutrients, medicinal and economic value, has become one of the most commercially important fish species in China [1]. More recently, with the increasing industrial farming of miiuy croaker, the fish has challenged many more diseases caused by bacteria, parasites and viruses than ever, resulting in severe infection diseases and economic losses. Hence, additional strategies in disease prevention and knowledge about the immune system are required for the establishment of effective measures in disease control [2,3]. For examples, to clearly identify the immune-relevant genes and pathways help to develop a strategy for establishment a successful genetic breeding program [4]. Previous studies had generated a non-normalized cDNA library and characterized a transcriptome with an emphasis on immune-relevant genes, as well as to identify potential molecular SSR and SNP markers [5,6]. On this basis, we studied deeply the host defense against viral infection depending on PRRs which expressed by cells of the innate immune system to identify PAMPs [7,8].

Innate immune response to virus infection has witnessed tremendous progress, especially in fish interferon antiviral response [9–12]. A line of fish genes involved in interferon antiviral response have been identified, and functional studies further reveal that fish possess an IFN antiviral system similar to mammals [13]. In fish virus-infected cells, type I IFN response is triggered through recognition of viral products by PRRs such as TLRs and RLRs [14,15]. In particular, TLR3-mediated and RIG-I/MDA5-mediated signaling pathway could eventually converge on the activation of IRF3 leading to the induction of type I IFN and subsequent ISGs, such as

Abbreviations: GO, Gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; DEGs, differentially expressed genes; ISGF3, IFN-stimulated gene factor 3; ISGs, IFN-stimulated genes; PRRs, host pattern recognition receptors; PAMPs, pathogen-associated molecular patterns; TLRs, toll-like receptors; RLRs, retinoic acid-inducible gene I (RIG-I)-like receptors; IRF3, interferon regulatory factor 3; MDA5, melanoma-differentiation-associated gene 5; LGP2, laboratory of genetics and physiology 2; RSEM, RNASeq by expectation maximization; FPKM, reads per Kilobase of exon model per million mapped reads; FDR, False Discovery Rate; KASS, KEGG Automatic Annotation Server; IPS-1, IFNβ promoter stimulator-1; TRIF, TIR-domain-containing adapter-inducing interferon-β; IKKe, inhibitor of kappaB kinase epsilon; TBK1, TANK-binding kinase 1; TRAF6, TNF receptor-associated factor 6; TRIF, TIR-domaincontaining adapter-inducing interferon-β.

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Mx1, PKR and ISG15 whose products can inhibit virus replication [16–18]. The TLRs (TLR3, 7, 8) which localized to the early endosomes can detect PAMPs, following activating interferon antiviral response [19,20]. Likewise, RLRs, a family composed of three cytoplasmic receptors: RIG-1, MDA5 and LGP2, recognize cytosolic viral component in majority of cell types to couple detection of infection by RNA viruses [21]. It is no doubt that RIG-I and MDA5 play as PPRs for viral PAMPs, while character of LGP2 in the antiviral response is debatable because LGP2 lack of CARDs domain that are conserved in proteins involved in immune signaling. MDA5 with its regulator LGP2, can recognize dsRNA such as poly(I:C), and subsequently enhances antiviral activity mediated by induction of type I IFN and IFN-stimulated gene expression [22].

In this study, we used poly(I:C) as a case study for dsRNA virus injection, and then examined the miiuy croaker compared transcriptomes. Subsequently, with the analysis of quantitative gene expression, differentially expressed genes (DEGs) in the comparison of the Ctrl and Poly libraries were picked up. What's more, we focused on screen out DEGs involving in immune defense with GO and KEGG enrichment analysis in detail. Finally, we emphatically analyzed the pathway map, RIG-I/MDA5-mediated and TLR3-mediated pathway, and the antivirus-related gene closely relating to dsRNA immune response.

2. Material and methods

2.1. Fish

Healthy miiuy croakers (mean weight 750 g) were purchased from Zhoushan Fisheries Research Institute (Zhejiang, China) and maintained in 1 m diameter fiberglass tanks supplied with recirculating freshwater at 25 °C [23]. After one week to acclimatize, fish were randomly divided into two groups, injection and control groups. Individuals in the injection group were challenged with 1 ml of diluted poly(I:C) (LMW, InvivoGen, 2.5 mg/ml) (designated as “Poly” sample), while the control groups were corresponding challenged with 1 ml physiological water (designated as “Ctrl” sample). The infected and control fish groups were respectively killed at 24 h after injection, and the spleen of the different individuals were removed and immediately kept at liquid nitrogen until RNA extraction [24].

2.2. Total RNA isolation, library construction and sequencing

Using TRIzol reagent (Qiagen), total RNA was extracted according to the manufacturer's instructions. Equal quantities of high-quality RNA was mixed with the same treatment condition from three different individuals. After the total RNA extraction and DNase I treatment, magnetic beads with Oligo (dT) were used to enrich the mRNA with that randomly broken into small pieces in fragmentation buffer. Whereafter, cDNA was synthesized using the mRNA fragments as templates, and the complementary strand was synthesized. In order to select the suitable fragments for PCR amplification, short fragments were purified, resolved with EB buffer for end reparation and connected with adapters. Libraries within the average length of ~300 bp were used for sequencing by Illumina HiSeq 2000 and sequencing progress was finished by Beijing Genome Institute (BGI).

2.3. Combined with genome-guided transcript and de novo for transcriptome assembly

Before data analysis, raw reads should be quality-filtered through removing adapter sequences, ambiguous nucleotides and the sequences whose quality is more than 20. And then, these remaining clean reads was used to subsequent assembly without redundancy. Afterward, we used two different pipelines, genome-guided transcript assembly and *de novo* assembly, to processing reliable transcriptome assembly. In detail, we firstly utilized TopHat [25] to align high-quality reads to miiuy croaker whole-genome (unpublished) and Samtools [26] to extract the alignments for properly paired-reads and unmapped reads. Subsequently, above-mentioned aligned reads were combined with reference transcripts to assembly the transcriptomes of poly and ctrl samples by Cufflinks [24]. Cuffmerge program in Cufflinks suite was performed to integrate the two samples transcripts into a single one. For the rest unmapped reads, Trinity [27] was used to transcriptome *de novo* assembly. Finally, we integrated the assembly results of genome-guided transcript and Trinity *de novo* assembly to get a comprehensive annotation of miiuy croaker for downstream analysis.

2.4. Identification of DEGs, within GO and KEGG enrichment analysis

Genes and isoforms expression level were quantified by a software package RSEM [28], and FPKM method was used in calculated expression level. To further elucidate the expression pattern of genes, we used the methods of the Poisson distribution that developed on a strict algorithm to identify DEGs comparing to two different samples. A statistical method FDR control was used to correct for multiple comparisons. When got FDR, we set the ratio of FPKMs of genes in two samples as fold change. Expressed genes with fold changes >2, FDR < 0.001 were defined as DEGs. After obtaining DEGs, we mapped all these genes against NCBI by using blastx with an E-value threshold of 1.0E-5. According to the result of NR annotation, we performed GO annotation by using the Blast2GO [29], and then used WEGO [30] to understand the distribution of gene functions of the species with p-value < 0.05 and FDR < 0.01 defined to the enrichment GO terms. Similarly, by mapping these DEGs to KEGG database via KASS, we identified significantly enriched pathway with Q-value $\leq$ 0.05 (corrected p-value) [31].

2.5. Evaluation of gene expression by real time PCR

To validate the accurate transcriptome, seven antiviral and immune-relevant genes involved in the RIG-I/MDA5-mediated signaling pathway concerned interferon antiviral response were used for expression analysis by qRT-PCR. RNA used for qRT-PCR amplifications were the same with those used to construct cDNA library for transcriptome. Total RNA was prepared from spleen injected with poly(I:C) using Trizol reagent in accordance with the manufacturer's instructions. cDNA was synthesized utilizing a QuantScript RT Kit according to the manufacturer's protocol, and then was stored at −20 °C for later. Real-time quantitative PCR was conducted as previously described by Xu et al. [32,33], and the primers of seven genes described as the Supplementary Table S1.

3. Results and disscussion

3.1. Transcriptome sequencing and assembly analysis

Two non-normalized transcriptome libraries were constructed and quality-filtered sequenced to acquire 5,03,39,252 clean reads in the Ctrl sample and 482,39,788 clean reads in the Poly sample. Subsequently, we used BWA to map these clean reads to whole-genome reference, and the statistics of alignment results were presented for each reference. The genome map rate of Ctrl and Poly samples were 93.93% and 94.06% respectively. and other detail