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Research paper

Evolutionary conservation and expression of *miR-10a-3p* in olive flounder and rock bream

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ABSTRACT

MicroRNAs (miRNAs) are small non-coding RNAs (ncRNAs) that mainly bind to the seed sequences located within the 3′ untranslated region (3′ UTR) of target genes. They perform an important biological function as regulators of gene expression. Different genes can be regulated by the same miRNA, whilst different miRNAs can be regulated by the same genes. Here, the evolutionary conservation and expression pattern of *miR-10a-3p* in olive flounder and rock bream was examined. Binding sites (AAAUUC) to seed region of the 3′ UTR of target genes were highly conserved in various species. The expression pattern of *miR-10a-3p* was ubiquitous in the examined tissues, whilst its expression level was decreased in gill tissues infected by viral hemorrhagic septicemia virus (VHSV) compared to the normal control. In the case of rock bream, the spleen, kidney, and liver tissues showed dominant expression levels of *miR-10a-3p*. Only the liver tissues in the rock bream samples infected by the iridovirus indicated a dominant *miR-10a-3p* expression. The gene ontology (GO) analysis of predicted target genes for *miR-10a-3p* revealed that multiple genes are related to binding activity, catalytic activity, cell components as well as cellular and metabolic process. Overall the results imply that the *miR-10a-3p* could be used as a biomarker to detect VHSV infection in olive flounder and iridovirus infection in rock bream. In addition, the data provides fundamental information for further study of the complex interaction between *miR-10a-3p* and gene expression.

1. Introduction

MicroRNAs (miRNAs) have been found to function as RNA regulatory genes in cells (Lim et al., 2003; Bartel and Chen, 2004; Bartel, 2009; Shukla et al., 2011). They are single-stranded RNAs (ssRNAs) (22 bp in length) that regulate expression levels of target mRNAs (He et al., 2005; Lund, 2010). MicroRNAs could be useful biomarkers for disease diagnosis and prognosis (Zhou et al., 2017; Correia et al., 2017). Several studies have demonstrated that miRNAs are associated with various diseases, including cancer and infectious diseases (Mao and Wang, 2015; Najib et al., 2017). They also perform important roles in several processes such as cellular proliferation, differentiation, development, apoptosis, and metabolism (Kren et al., 2009; Lelandais-Briere et al., 2010; Mach et al., 2013; Ali et al., 2013).

The miR-10 family has been retained within the homeobox (Hox) clusters in mammals, birds, reptiles, and fishes (Lemons and McGinnis, 2006; Yekta et al., 2008; Woltering and Durston, 2008). Hox genes encode transcription factors for the homeobox domain capable of

Abbreviations: miRNAs, MicroRNAs; ncRNAs, non-coding RNAs; 3′ UTR, 3′ untranslated region; VHSV, viral hemorrhagic septicemia virus; GO, gene ontology; ssRNAs, single-stranded RNAs; Hox, homeobox; GCSF, granulocyte colony stimulating factor; *S. parauberis*, *Streptococcus parauberis*; CFU, colony forming units; PFU, plaque-forming units; RBIV, rock bream iridovirus; PNU-IACUC, Pusan National University-Institutional Animal Care and Use Committee; HB, HeimBiotek; cDNA, complementary DNA; RT, Reverse Transcription; RT-PCR, Reverse Transcription Polymerase Chain Reaction; ECR browser, Evolutionary Conservation of Genomes; PANTHER, Protein ANalysis THrough Evolutionary Relationships; GGI, gene–gene interaction; PPI, protein-protein interaction; HoxC5, Homeobox C5; PBX, Pre-B-cell leukemia transcription factor; NFE2L, Nuclear factor (erythroid-derived 2)-like 2; MLL, mixed-lineage leukemia; C19MC, chromosome 19 microRNA cluster; Cdc42, Cell division control protein 42 homolog; EPC, epithelioma papulosum cyprinid; hpi, hours post infection; ADAR, Adenosine deaminases acting on RNA; HCV, hepatitis C virus

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binding to the DNA. Hox proteins regulate transcription through a number of different binding sites (Pearson et al., 2005). Hox genes show complex patterns of polycistronic transcription by alternative transcription start sites (Hadrys et al., 2004). The Hox clusters produce several types of ncRNAs including miRNA (miR-10a, miR-10b, miR-196a, miR-196b) in mammals and fishes (Tanzer et al., 2005; Rinn et al., 2007; Woltering and Durston, 2008; Yekta et al., 2008). The Hox cluster-embedded miRNAs preferentially target Hox mRNA, and Hox transcription factors confer anterior-posterior axial coordinates to vertebrate embryos (Yekta et al., 2008). The retention of miR-10 and miR-196 within Hox clusters could be explained by their proximity to other coding genes, leading to a coordinated expression pattern of the miRNA and their targets. Hox miRNAs participate in biological functions as delayed negative post-transcriptional regulators of their 3′ Hox gene targets (Woltering and Durston, 2008; Stark et al., 2008). The miR-10 knockdown resulted in upregulation of the target genes and overexpression of miR-10 induced phenotypes associated with loss of HoxB1a and HoxB3a in zebrafish (Woltering and Durston, 2008). Phylogenetic evolution of multiple miRNA family was examined in the Japanese flounder (Fu et al., 2011). Recently, the expression profile of miRNAs in response to viral hemorrhagic septicemia virus (VHSV) infection was presented in the olive flounder (Najib et al., 2016). Protective immune responses of granulocyte colony stimulating factor (GCSF) in rock bream during *Streptococcus iniae* and iridovirus infection have been reported (Jeswin et al., 2017). In this study, the evolutionary conservation and expression pattern of miR-10a-3p was examined in various tissues of olive flounder and rock bream.

2. Materials and methods

2.1. Streptococcus parauberis, VHSV, and RBIV infection

Olive flounder (*Paralichthys olivaceus*) was subcutaneously injected with *S. parauberis* at a dose of 5.06×10^3 colony forming units (CFU)/fish, and intramuscularly injected with the viral hemorrhagic septicemia virus (VHSV) at a dose of 10^6 plaque-forming units (PFU)/fish. After seven days, gill tissues of olive flounder infected by *S. parauberis* and VHSV were collected for isolation of RNAs. Spleen, liver, kidney tissues (10^7 copies/mg) of rock bream (*Oplegnathus fasciatus*) naturally infected by rock bream iridovirus (RBIV) were also gathered for further RNA isolation.

2.2. RNA samples from various tissues of olive flounder

The dissection of various tissues was carried out in compliance with the guidelines of the Pusan National University-Institutional Animal Care and Use Committee (PNU-IACUC). Total RNAs from various tissues (brain, muscle, liver, spleen, gill, kidney, intestine, fin, stomach, and testis) of olive flounder and rock bream were extracted using TRIzol reagent (Invitrogen) with RNase-free DNase I (New England BioLabs) according to the manufacturer's protocol. Each RNA samples were quantitated to 500 ng using a NanoDrop® ND-1000 UV–vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

2.3. Quantitative RT-PCR amplification

Each of the RNA samples was prepared with reaction volume of 20 μL. The HB miR Multi Assay Kit™ System I (HeimBiotek, Korea) was used for the analysis of miRNA in a 2-step process. In the initial step—synthesis of complementary DNA(cDNA)—HB_I Reverse Transcription (RT) Reaction Kit and its reagents was used according to manufacturer's suggestions and then Reverse Transcription Polymerase Chain Reaction (RT-PCR) amplification was performed in a thermal cycler (Eppendorf, Hamburg, Germany), at conditions of 37 °C for 60 min (Step 1) followed by incubation at 95 °C for 5 min (Step 2) for 1 cycle and then held at 4 °C. This gave a final product of cDNA for each respective total RNA sample with a total volume of 20 μL respectively. The final product was stored at − 20 °C until further use.

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>hsa-miR-10a-3p (Human)      MIMAT0004555 CAAAUUCGUAUCUAGGGGAAUA
>mml-miR-10a-3p (Rhesus)     MIMAT0026797  AAAUUCGUAUCUAGGGGAAUA
>chi-miR-10a-3p (Goat)       MIMAT0035912 CAAAUUCGUAUCUAGGGGAAU
>oan-miR-10a-3p (Duckbill)   MIMAT0007123 CAAAUUCGUAUCUAGGGGAAU
>ssc-miR-10a-3p (Pig)        MIMAT0022954 CAAAUUCGUAUCUAGGGGAAU
>mmu-miR-10a-3p (Mouse)      MIMAT0004659 CAAAUUCGUAUCUAGGGGAAUA
>rno-miR-10a-3p (Rat)        MIMAT0004709 CAAAUUCGUAUCUAGGGGAAUA
>cgr-miR-10a-3p (Hamster)    MIMAT0023736 CAAAUUCGUAUCUAGGGGAAU
>mdo-miR-10a-3p (Marsupial) MIMAT0026648  AAAUUCGUAUCUAGGGGAAUA
>gga-miR-10a-3p (Chicken)    MIMAT0007732  AAAUUCGUAUCUAGGGGAAUA
>oha-miR-10a-3p (Cobra)      MIMAT0036661 CAAAUUCGCAUCUAGGGGAAUA
>aca-miR-10a-3p (Lizard)     MIMAT0021718  AAAUUCGCGUCUAGGGGAAU
>ssa-miR-10a-3p (Salmon)     MIMAT0032293 CAAAUUCGUGUCUAAGGGAGU
>bfl-miR-10a-3p (Lancelet)   MIMAT0009467 CAAAUUCGUUUCUGCAGGUAU
>dre-miR-10a-3p (Zebrafish) MIMAT0003391 CAAAUUCGUGUCUUGGGGAAUA
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Fig. 1. Evolutionary conservation of miR-10a-3p in various species. The binding sites (AAAUUC) for the seed region of the 3′ UTR target genes were highly conserved.

For the second step in the two-step process, HB_I Real-time PCR Master mix kit from the HB miR Multi Assay Kit™ System I was used according to manufacturer's suggestions in Rotor-Gene Q system (QIAGEN, Hilden, Germany). The amplification protocol was performed as follows: initial denaturation for 15 min at 95 °C; 45 thermal cycles of 95 °C for 10 s and 60 °C for 40 s; standard melting conditions of ramp ranging from 55 °C to 99 °C with 1 °C rise on each step. Micro RNA U6 was used as reference. The results were analyzed as a relative expression ratio of the target miR-10a-3p (5′-CAAAUUCGUAUCUAGGGGAAUA-3′) to miRNA U6 using the comparative threshold method (2-ΔΔCt). All samples were performed in triplicate and the mean values of the resulting relative expression ratios were used for analyses and generation of charts.

A PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa, Kyoto, Japan) was used for cDNA synthesis and gDNA removal from 0.5 μg of total RNA. The quantitative real-time PCR primers for HOXC4 gene were designed by using Primer 3 program. The sense primer is made up of (5′-AGCATCCAAACAGCACAGC-3′), and the anti-sense primer is made up of (5′-CTCCGCCGAGTTAAATATCG-3′). The amplification protocol was performed as follows: initial denaturation for 15 min at 95 °C; 45 thermal cycles of 59 °C for 15 s and 72 °C for 15 s; standard melting conditions of ramp ranging from 55 °C to 99 °C with 1 °C rise on each step.

2.4. Bioinformatic analyses of miR-10a-3p and its target genes

The sequences of mature miR-10a-3p members were identified via miRBase v19.0 (http://www.mirbase.org), and evolutionary conservation of those miRNAs was analyzed using the Evolutionary Conservation of Genomes (ECR browser) (https://ecrbrowser.dcode.org/). Target gene prediction for the miR-10a-3p was performed using TargetScan 7.1 (http://www.targetscan.org/vert_71/). The functional enrichment analysis of those target genes was conducted using the Protein ANalysis THrough Evolutionary Relationships(PANTHER) classification system (http://www.pantherdb.org/). GeneMANIA (http://genemania.org/) and the STRING database (http://string-db.org/) were used to identify the gene–gene interaction (GGI) network and protein-protein interaction (PPI) network, respectively. The GGI network was composed of seven criteria (physical interaction, co-expression, predicted, pathway, co-localization, genetic interaction, and shared protein domain) from the GeneMANIA database. The PPI network was examined based on four criteria (experiment, database, textmining, and coexpression) from the STRING database.