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Immune-related genes response to stimulation of miR-155 overexpression in CIK (ctenopharyngodon idella kidney) cells and zebrafish

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ABSTRACT

MiR-155 regulates the development of germinal-center and the generation of immunoglobulin class-switched plasma cells. However, whether miR-155 is involved in immune response in fish is still unclear. Here, CIK cells transfected with miR-155 overexpressed plasmid inhibited mRNA expression of mIg and Rag2 ($P < 0.05$). Interestingly, mIg was predicted as a potential target gene of miR-155 by RNAhybrid, with a putative binding site in its CDS. Further, mIg luciferase reporter vectors with successive deletions of mIg cDNA sequence were constructed and dual luciferase reporter assay showed that vectors containing the sequence from 318 to 347 in CDS exhibited lower relative luciferase activity than others without predicted binding region ($P < 0.05$), which indicated mIg is the target gene of miR-155 and reveal bona fide targeted binding site of mIg for miR-155 in fish. In vivo, the zebrafish were respectively injected with miR-155 overexpressed and empty vector, and showed that miR-155 efficiently expressed in zebrafish ($P < 0.01$), which consistently decreased mRNA level of immune-related genes, including *mIg* ($P < 0.01$), *sIg* ($P < 0.05$), *AID* ($P < 0.01$), *PU.1* ($P < 0.05$) and *Rag2* ($P < 0.05$) at d 3 and d 6 post injection, comparing to control. Collectively, this work indicates that overexpression of miR-155 suppresses the mRNA level of immune-related genes in CIK cells and zebrafish, and mIg is a novel target gene of miR-155 in fish. These findings provide an insight into the miR-155 modulating adaptive immunity in grass carp and zebrafish.

1. Introduction

Regulation of the adaptive immune system (AIS) is necessary for jawed fish to protect from the pathogen invasion. This process is a highly specialized and tightly regulated, involving in the interaction of both lymphocytes and immune-relevant molecules [1], like *Rag2* (the recombination activating genes), *AID* (activation-induced cytidine deaminase), *TCRAC* (TCR alpha constant domain), *mIg* (immunoglobulin M heavy chain membrane form) and *sIg* (immunoglobulin M heavy chain secretory form) [2]. Of them, the mIg is found on the surface of early or memory B cells and has a longer and highly hydrophobic C-terminal segment, which is essential for anchoring the receptor molecules to the lipid bilayer [3]. However, little is known about the post-transcriptional regulation (miRNA) on *mIg*.

MicroRNAs (miRNA), small untranslated RNA species, are capable of degrading or silencing messenger RNA (mRNA) target by binding its 3′-untranslated region (UTR) [4,5]. Interestingly, some targeted sites of miRNAs locate outside of 3′-UTR in mRNA, including coding regions (CDS) and 5′-UTR [6], which suggest that the functional molecular mechanism of miRNA is complex. Recently, knockout the entire miRNA regulatory network typically by Cre-Loxp mediated excision of genes (*Dicer*, *Drosha* or *Argonaut*), is involved in B cell differentiation from the pre-B cell stage onwards [7], sustained terminal deoxynucleotidyl transferase expression, altered generation of the antibody repertoire [8] and reduced in total thymocyte numbers [9]. These indicate that miRNAs network plays critical roles in multiple faucets of adaptive immunity, from the development of the key cellular players to the activation and function of the immune responses [10]. However,

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Table 1
Real-time qPCR primers (S sense, A antisense, RT reverse transcript) for genes.

Gene	Primer	Sequence (5′–3′)	Product length	Tm	Accession Number
mIg (zebrafish)	SA	ATGCCTGGATTGTGTGGATAGAGCCACCTTAACAAAAGTAGCACCTATGC	143 bp	60 °C	AF281479.1
mIg (grass carp)	SA	TCAATCTTCGCCCTGTCTCAGGGTGGTGGAGTTGGAGGTA	177 bp	60 °C	DQ417927.1
sIg (zebrafish)	SA	GAGACCGTCAGTTTACGTGTTAGCAAGTCACAAACACCTCCTTGGGC	107 bp	60 °C	AF281480.1
PU.1 (zebrafish)	SA	ATCCCAGCAGTCGTAGTCCTTCTCGATCCACCCACCAGAT	140 bp	60 °C	NM_001328369.1
Rag2 (zebrafish)	SA	GACCTAGAGTTTGGCTGCTGTACGCATGGAGACGGATCAAACGAGAG	157 bp	56 °C	NM_131385.3
Rag2 (grass carp)	SA	CCATAGCCGAGGGAAGACCGATGTGACCGCCCAGAAAGT	235 bp	60 °C	FJ494965.2
AID (zebrafish)	SA	GTAGAGCTTCTCTTTCTGCGTCACAGCAGAACCAGGTCACTGAGTAAC	103 bp	56 °C	NM_001008403.1
β-actin (zebrafish)	SA	CGAGCAGGAGATGGGAACCCAACGGAAACGCTCATTGC	102 bp	60 °C	NM_131031.2
miR-155	RTSA	GTCGTATCCAGTCGAGGGTCCGAGGTATTCGCACTGGATACGACCCCCTACGCCGTTAATGCTAATCGTGATAGCGAGGGTCCGAGGTATTCCG	69 bp	60 °C	LM609208.1
18S rRNA (grass carp)	SA	GGACACGGAAAGGATTGACAGCGGAGTCTCGTTCGTTATCGG	120 bp	60 °C	NR_145818.1

exploring the specific miRNA and its target, is facilitated to elucidate the underlying mechanism of miRNAs on adaptive immune responses and establish the miRNAs network during this process.

The miR-155 is processed from the noncoding RNA transcribed from *Bic* (B cell integration cluster), which is highly expressed in lymphocytes and mediates lymphocyte proliferation, differentiation and subgroup drifting [11–16]. Recently, research has indicated that miR-155 regulates the development of germinal-center and the generation of immunoglobulin class-switched in mammal [17,18]. However, the validated roles and targeted genes of miR-155 are unknown in CIK cells and zebrafish, which have been established as an attractive model for the study of development of the immune system [19]. Based on the sequence alignment of mature miR-155, the sequence in zebrafish is not completely consistent with that of mouse [20], therefore the effects of miR-155 in fish adaptive immune need to be identified.

Here, overexpression of miR-155 in CIK cells inhibited mRNA level of immune-related genes (mIg and Rag2). Interestingly, mIg was identified as a novel target gene of miR-155 and the bona fide binding site within its CDS was revealed in CIK cells by the dual luciferase reporter assay. Further, miR-155 overexpressed in vivo (zebrafish) significantly decreased the mRNA levels of *mIg*, *sIg*, *AID*, *PU.1* and *Rag2* at d 3 and d 6 post injection. These findings suggested that miR-155 inhibits the expression of immune-related genes in CIK cells and zebrafish, and mIg is the target of miR-155.

2. Materials and methods

2.1. Cell culture and plasmids transfection

CIK cell line (provided by Prof. Jianguo Su from Huazhong Agricultural University) was grown in DMEM (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, Hyclone, USA), 100 IU/ml of penicillin (Sigma, USA) and 100 IU/ml of streptomycin (Sigma) [21]. Cells were incubated at 28 °C in a 5% CO_2 humidified atmosphere. CIK cells were transfected in 6-well plates at a density of 2×10^6–5×10^6 cells/ml with 1 μg of pAd-CMV-miR-155 plasmid (as miR-155) or empty vector (as Control) by FuGENE® HD Transfection Reagent (Roche, Switzerland) accordingly to the manufacturer's instruction.

2.2. Fish

Zebrafish (average length is around 3 cm) were purchased from Changxing fish farm (Xianyang city, Shaanxi province, China). The fish were maintained in several 30 L aquarium tanks under laboratory conditions (oxygen content higher than 85% saturation, 24 ± 1 °C, fed with egg yolk granules diet every morning).

2.3. Experimental injection and sampling procedures

The plasmids injection and sampling procedures were performed as described [22]. In briefly, each fish was injected 10 μl of total 2 μg control or overexpressed vectors. The fish were divided into three groups: untreated, empty vector injected (as control) and miR-155 overexpressed plasmid injected (as miR-155). At d 0, d 3, and d 6 post injection, 4 individuals from each group were randomly sampled, respectively. The kidney of each sampled fish was crushed in a mortar filled with liquid nitrogen and stored at −80 °C.

2.4. RNA isolation, cDNA synthesis

Total RNA was isolated using TRIzol reagent (Takara, Japan) following the manufacturer's instructions. The extracted RNA was digested by RNase-free DNase I (Takara, Japan) to remove genomic DNA contamination. The RNA concentration in each sample was quantified using a spectrophotometer at 260 nm and the purity of RNA was

assessed by measuring OD260 nm/OD280 nm ratio (range 1.90–2.05). The integrity of RNA was checked by electrophoresis on 1.0% agarose gels with ethidium bromide staining. Two micrograms of total RNA from each category as well as control was reverse transcribed to obtain cDNA using MMLV reverse transcriptase (Takara, Japan) with oligo (dT) 18 primer and miR-155 stem-loop primer following the manufacturer's protocol, respectively. All the RNA and cDNAs samples were stored at −80 °C until used.

2.5. Real-time qPCR

Gene expression profiles were evaluated using real-time qPCR. The primers for different immune response genes and β-actin (reference gene) were obtained in previous studies [22] and listed in Table 1. Real-time qPCR reactions were carried out in a final volume of 25 μl, using SYBR Premix Ex Taq (TaKaRa, Japan), 0.4 mM of each primer, and 200 ng of cDNA template. Each individual sample was run in triplicate wells. PCR amplification cycles were performed using iQ™5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA). The melting curve analysis was performed after amplification to verify the accuracy of each amplicon. All tested genes mRNA levels were given by the formula $F = 2^{-\Delta\Delta Ct}$ and reported as fold change in abundance relative to the average control response.

2.6. Construction of luciferase reporter vectors and detection of luciferase activity

The fragments of mIg were amplified by the primers (Table 2) and then cloned into siCHEK™-2 vector at NotI and XhoI sites. The intended sequences were confirmed with sequencing. The dual luciferase assay was performed as described [23]. CIK cells were plated in a 24-well plate at a density of 4×10^4 cells/well in 500 μl culture medium 24 h prior to transfection; the cells were then co-transfected with the psiCHECK™-2 reporter plasmid and pAd-CMV-miR-155 or empty vectors using TurboFect Transfection Reagent (Thermo Scientific, USA) accordingly to the manufacturer's recommendations. Renilla and firefly luciferase activities were measured at 24 h after transfection using a dual luciferase kit (Promega, USA). The relative luciferase activity was indicated by the ratio of firefly luciferase activity and renilla luciferase activity.

2.7. Statistical analysis

All data were expressed as mean ± S.E. and were analyzed by ANOVA with Dunnett's test after normalization (SPSS 16.0 software). The differences were considered significant at $P < 0.05$, and extremely significant at $P < 0.01$.

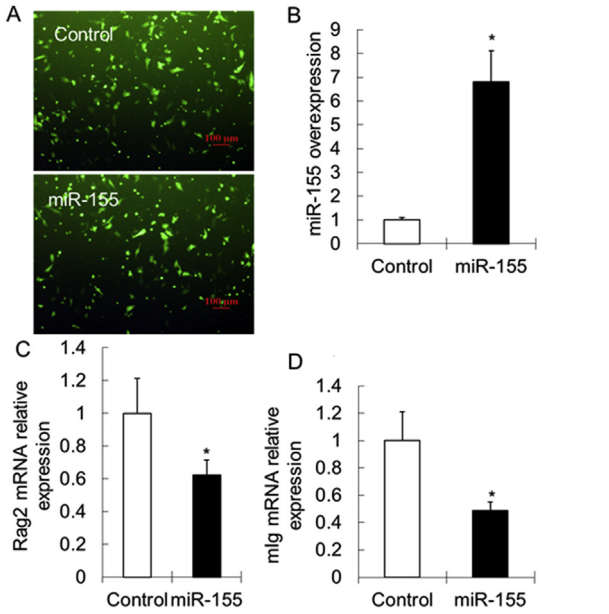

Fig. 1. Overexpression of miR-155 decreases mRNA expression of mIg and Rag2 in CIK cells. (A) The GFP expression after transfected plasmids in CIK cells. (B) The expression of miR-155 after transfection. (C–D) The relative mRNA expression of mIg and Rag2, respectively. N = 4, *$P < 0.05$.

3. Results

3.1. Overexpression of miR-155 inhibits expression of mIg and Rag2 in CIK cells

To identify the immune response of miR-155 overexpressed in vitro, the CIK cells were transfected with pAd-cmv-miR-155 or empty vectors. The expression of GFP (Fig. 1A) in these cells suggested that this transfection was efficient. Consistently, qPCR result showed miR-155 transfected group with dramatically higher ($P < 0.05$) level of mature miR-155 than that of control (Fig. 1B). Interestingly, miR-155 overexpressed significantly decreased the mRNA levels of *mIg* and *Rag2* ($P < 0.05$) to ~60% and ~40% of the control, respectively (Fig. 1C and D). The findings suggest that miR-155 inhibits the expression of immune-related genes (*mIg* and *Rag2*) in CIK cells.

3.2. The mIg is a target gene of miR-155

To analyze the underlying mechanism of miR-155 downregulation of above detected adaptive immune related genes, we used online target prediction algorithm to predict potential binding sites within the immune-related genes, based on the criteria of miRNA binding 3′-UTR or coding region of target genes (http://bibiserv.techfak.uni-bielefeld.de/rnahybrid/submission.html). Consequently, one binding site within CDS of *mIg* and 3′-UTR of *AID* was predicted (Fig. 2A and Fig. S1). Previous research have reported that AID was the target gene of miR-

Table 2
Primer (S sense, A antisense) for constructing the luciferase reporter vector.

Gene	Primer	Sequence (5′–3′)	Product length	Tm
F1R1	S	CCGCTCGAGGTTCATATTGACATCATCCCTCC	317 bp	62 °C
	A	ATTTGCGGCCGCTCAAACAGAGGATGCTCTATC		
F1R2	S	CCGCTCGAGGTTCATATTGACATCATCCCTCC	353 bp	62 °C
	A	ATTTGCGGCCGCGTGTTTGCAATGCCACTGTC		
F1R3	A	CCGCTCGAGGTTCATATTGACATCATCCCTCC	539 bp	60 °C
	S	ATTTGCGGCCGCTGAAAATCTAACAACAAATACCAA		
F2R3	A	CCGCTCGAGTTCCTGTTCCTCATCACCTTG	190 bp	60 °C
	S	ATTTGCGGCCGCTGAAAATCTAACAACAAATACCAA		

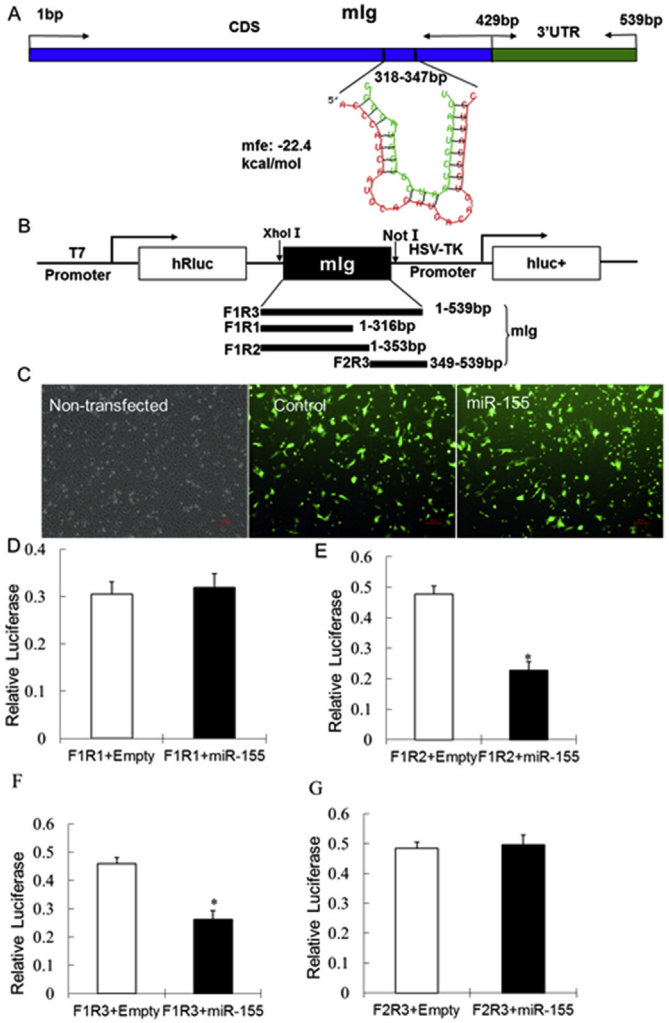

Fig. 2. MIg is the target gene of miR-155 in CIK cells. (A) The putative binding site of miR-155 within mIg mRNA was predicted by RNAhybrid. The sequence marked in red is mIg CDS, and sequence marked in green is the mature sequence of miR-155. mfe: minimum free energy. (B) Schematic representation of the plasmids used in the luciferase assay. (C) The GFP expression in CIK cells after co-transfected with pAd-CMV-Control/pAd-CMV-miR-155 and psiCHECK-2-mIg plasmids. (D–G) Measurement the dual luciferase activity at 36 h post transfection. N = 4, $*P < 0.05$, compared with control. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

155 regulating immune response [24]. Thus, we next constructed the psiCHECK-2-mIg luciferase reporter vectors with successive deletions of mIg cDNA related sequence (Fig. 2B) to reveal the bona fide binding site of miR-155 within mIg mRNA sequence. The dual luciferase reporter assay showed that relative luciferase activity decreased in F1R2 and F1R3 transfected group (Fig. 2C, E and F), including the predicted binding site (318–347 bp), but not in F1R1 and F2R3 group without this region (Fig. 2D and G). The results indicate that *mIg* is a target gene of miR-155, and the binding site is from 318 to 347 of *mIg* CDS.

3.3. miR-155 is highly expressed in zebrafish by plasmid injection

To explore the immune function of miR-155 in vivo, we injected overexpression plasmids into zebrafish, and randomly selected 4 individuals from each group at day 3 post injection, smeared its kidney tissue onto a glass slide, and then observed GFP signal using the fluorescence microscope. As observed, GFP was shown in both the control and miR-155 groups, not in the untreated group (Fig. 3A).

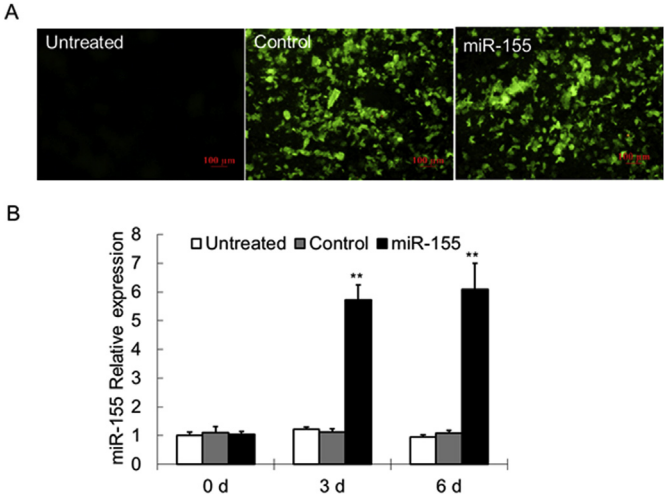

Fig. 3. miR-155 is expresses efficiently by vectors injection in zebrafish. (A) Kidney tissue smears of zebrafish injected with empty plasmid and pAd-CMV-miR-155 vectors under a fluorescence microscope at d 3 post injection. (B) Detection of miR-155 by qPCR at d 0, d 3 and d 6 after injection. N = 4, $**P < 0.01$.

Furthermore, qPCR analysis showed that miR-155 overexpression group had significantly higher level of miR-155 at d 3 and d 6 post injection (Fig. 3B), whose level reached to ~6 and ~7 fold changes to that of control or untreated groups ($P < 0.05$). These results indicated that the miR-155 efficiently expresses in zebrafish through injection with miR-155 overexpressed plasmid.

3.4. Overexpression of miR-155 suppresses immune-related genes in vivo

Next, the total RNA extracted from kidney tissue were carried out qPCR to validate the effects on expression of immune-related genes after injected with control and miR-155 overexpression plasmid in zebrafish. Firstly, the results showed that the mRNA levels of the immune-related genes gradually increased in the empty plasmid injected group from d 0 to d 6 comparing to the untreated group (Fig. 4), including *mIg* with ~7 fold, *sIg* with ~2.5 fold, *PU.1* with ~6 fold, *AID* with ~5 fold, *Rag2* with ~3.5 fold and *TCRAC* with ~35 fold changes at d 6 post injection, which interestingly indicates that the zebrafish has immune response by plasmid injection. In parallel with the data of in CIK cells, overexpression of miR-155 dramatically decreased mRNA level of mIg (Fig. 4A), compared with that of control. Consistently, other adaptive immune related genes of *sIg*, *AID*, *PU.1* and *Rag2* exhibited a significant decrease at d 3 and d 6 after injection ($P < 0.05$), respectively (Fig. 4B, C, Fig. 4D and E). Moreover, the mRNA expression of *TCRAC* showed a decreased trend at d 3 and also went through a significant decrease ($P < 0.01$) at d 6 post injection (Fig. 4F). Collectively, overexpression of miR-155 down-regulates the expression of adaptive immune-related genes in vivo.

4. Discussion

In this study, we found that overexpression of miR-155 in CIK cells inhibits expression of mIg and Rag2. Interestingly, mIg is a novo target of miR-155 and the exactly binding site is identified. Similarly, miR-155 plasmid injection into zebrafish decreases the mRNA level of adaptive immune related genes in vivo. As CIK cells is widely used to study immune response and anti-invasion in vitro [25] and Zebrafish is usually selected as the animal model because of its with small body size, rapid growth and development, as well as its advantages about immunity study [26]. Thus, these results provide an insight into the miR-155 modulating immunity response in fish.

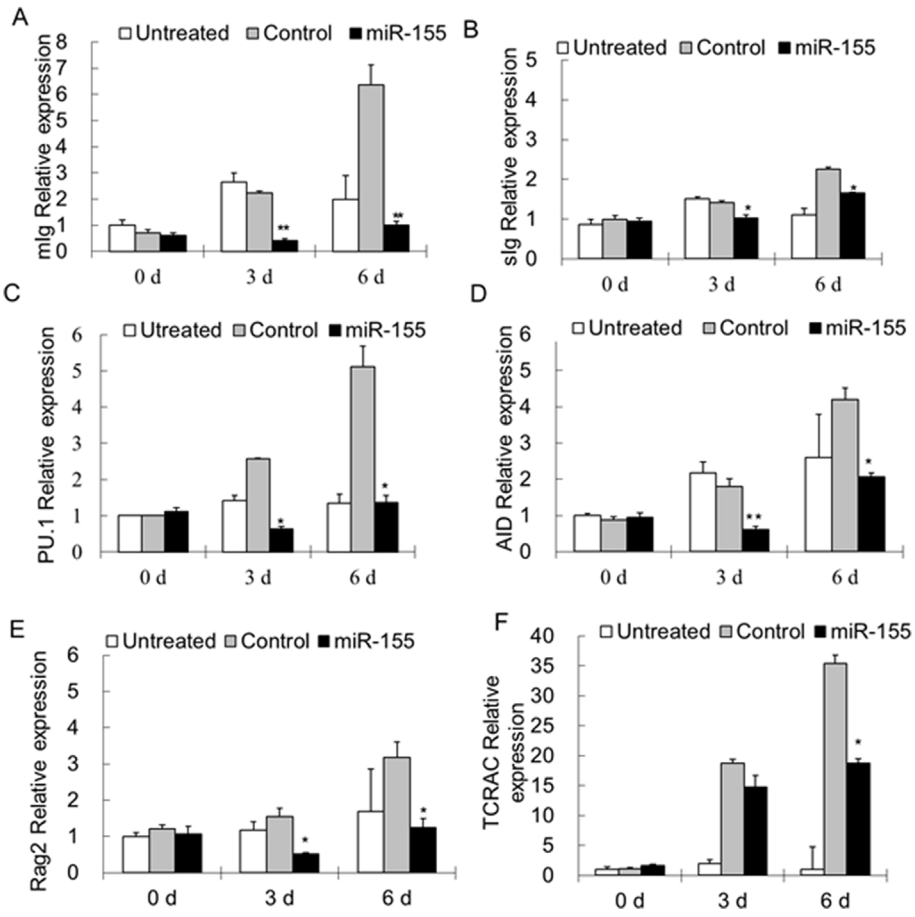

Fig. 4. miR-155 overexpressed inhibits expression of adaptive immune related genes in zebrafish. (A–F) Expression of (A) mIg, (B) sIg, (C) PU.1, (D) AID, (E) Rag2, (F) TCRAC genes relative to β-actin gene in zebrafish at d 0, d 3 and d 6 after injection. Transcript abundance is expressed relative to that of the control. N = 4, $*P < 0.05$, $**P < 0.01$.

We found that overexpression of miR-155 inhibits mIg in grass carp kidney cell line (CIK) and zebrafish. *mIg* attaches to the surface of B cell and is regarded as a B cell receptor (BCR) [27], which is involed in antigen recongnising the B cell to induce its maturation, subsequently might affect immunoglobulin production. Previous research reported that mIg and sIg mRNAs originate from a single transcript (pre-mRNA) by differential alternative processing, which occurs in nucleus [28]. Our results indicate that mIg is a target of miR-155. Interestingly, miR-155 overexpression also downregulates the mRNA level of sIg in zebrafish. Recently, studies have revealed that mature miRNAs can be transported from the cytoplasm to the nucleus to recognize target gene [29,30]. Moreover, miR-155 is detected in nucleus by deep sequence in human [31,32]. Thus, we speculate that partial miR-155 might targeting common pre-mRNA of mIg and sIg in nucleus partly contribute to decreasing both mRNA level of mIg and sIg.

In general, miRNA can complementarily bind to the mRNA 3′UTR region to regulate the gene expression by transcriptional repression or mRNA degradation [33,34]. Interestingly, our results showed that miR-155 binds to CDS of mIg located from 318 to 347. For CDS targets of miRNAs, there are still debates. Some research reported that miRNAs do not mediate mRNA posttranscriptional regulation through CDS-located sites [35], whereas more and more evidences suggested that miRNAs induce mRNA degradation or translational repression through CDS-located sites [6,36]. Additionally, Liu GJ et al. found that CDS-located target sites are more conserved compared with nontarget control, suggesting a stronger functional constraint due to miRNA binding to CDS region regulation [37]. Thus, our study not only supports an augmented model in which animal miRNAs can regulate mRNAs by binding to targets outside as well as inside the 3′UTR [36,38–41], but also extends our understanding of how miR-155 regulates immune response in fish.

This study also found that overexpression of miR-155 inhibits other immune-related genes, including *PU.1*, *AID* and *TCRAC* in zebrafish. As no annotation of these genes in grass carp, whose expression were not detected in CIK cells. Of them, PU.1 plays a key role in the development of immune system [22]. It's reported that miR-155 down-regulates the mRNA level of *PU.1* by targeting its 3′UTR in mammal, contributing to the generation of Ig class-switched plasma cells [42]. However, we did not find the putative binding site of miR-155 within *PU.*1 mRNA in zebrafish. The possible explanation is that miR-155 targets other transcriptional factors indirectly regulating *PU.1* expression, such as *C/EBPβ*, a classical target of miR-155 [43]. In mice, *C/EBPβ* is a target gene of miR-155 in B-cell [44] and other cell types [43,45], which has been proved to regulate the transcription of *PU.1* [46]. Nevertheless, regarding absence of RNA sequence annotation of *C/EBPβ* in fish, this speculation need further study.

In addition, our results have showed that overexpression of miR-155 also down-regulates the mRNA level of *AID,* which cause mutations to produce antibody diversity in B cell [47]. Previous research indicated that AID could be induced by transcription factors, such HoxC4 (Homeobox C4), Irf8 (Interferon regulatory factor 8) and Pax5 (Paired box gene 5) [48]. At the post-transcriptional level of regulation, AID mRNA level is also deceased by miR-155 in mice [49]. It's reported that disruption of the miR-155 binding site in the 3′UTR of *AID* resulted in up-regulating *AID* expression, which is similar as the effect of miR-155 deficient mice [24]. Therefore, we speculate that the inhibition of expression of *AID* might be a result of miR-155 directly binding to 3′UTR of *AID* in fish.

In summary, we found that miR-155 overexpression down-regulates mRNA expression of *mIg* and *Rag2*, and *mIg* is identified as a novel target gene of miR-155 in CIK cells. Moreover, miR-155 overexpression decreases mRNA expression of *mIg*, *sIg*, *AID, PU.1*, *Rag2* and *TCRAC* in

zebrafish. These findings provide a theoretical foundation to further understanding the modulation of miR-155 on adaptive immunity in zebrafish and grass carp.

Declaration of interest

The authors declare no financial and non-financial competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.002.

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