MiR-1397 Regulates Chicken CARP Gene Expression at Post-transcriptional Level

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Abstract

Objectvie To identify whether miR-1397 specific targeting chicken carp gene and further analyze its expression pattern under different conditions. Methods Using online softwares, we predict a potential binding site for miR-1397 in chicken carp 3' untranslate region (3'-UTR). Then further confirmed miR-1397 specifically regulate the expression of chicken carp gene by luciferase activity analysis, site-directed mutagenesis, Real time PCR and Western blot. Simultaneously, Realtime PCR was used to analysis the expression of miR-1397 in chicken embryonic myoblasts (CFM) at different developmental stage and in different tissues of adult chicken. Results The miR-1397 can downregulate the activity of carp 3'-UTR luciferase reporter gene and inhibit the expression of endogenous carp, while the binding site mutation in carp 3'-UTR significantly reduced the inhibitory effect of miR-1397. In addition, in the CFM cell differentiation process, the expression of carp increased, while the expression of miR-1397 was decreased. Conclusion MiR-1397 can regulate the expression of chicken carp at post-transcriptional level.

Keywords

Chicken; carp; miR-1397; post-transcriptional regulation.

1. Introduction

Cardiac ankyrin repeat protein (carp) is specifically expressed in mammalian cardiac muscle[1] and plays important roles in transcriptional regulation[2], the cytoskeletal architecture[3], and stretch sensing during heart development and pathological processes[4-8]. In contrast to its mammalian homologs, our previous study found that the expression of chicken carp is restricted to skeletal muscle and could be down-regulated by Myostatin[9,10]， which a main negative regulator of muscle growth[11-13]. Moreover, our recent functional study demonstrated that carp may play a key role in the myostatin signaling cascade that controls chicken skeletal myogenesis through inducing proliferation and avoiding apoptosis during CFM cells differentiation[10]. However, no reports were shown to investigate chicken carp's expression regulatory mechanisms.

MicroRNAs (miRNAs) are known to negatively regulate the expression of their target genes[14] and have an important role in myogenesis[15]. Given the large size of the chicken carp 3'-untranslation region (3'-UTR) (2.2 kb)[10], we hypothesized that it was likely to be targeted by miRNA. Therefore, we used bioinformatics to predict miR-1397 that would target the carp 3'-UTR, and validated the predictions using quantitative real-time PCR and western blot assays to confirm expression of the miR-1397 in muscle, as well as transfections with exogenous pre-miR-1397 to confirm targeting of the carp 3'-UTR. Moreover, during chicken embryonic myoblasts (CFM) cell differentiation, the expression of carp increased, while the expression of miR-1397 decreased correspondingly. Therefore, we suggest that inhibition of translation by miR-1397 is an important mechanism regulating carp expression and that it could be targeted for improving meat yield in poultry production.

2. Materials and methods

2.1 Experimental materials

Receptive Escherichia coli DH5 alpha, Lipofectamine 2000 transfection reagent, RIPA lysate purchased from Guangzhou Ding Guo Biotech Corp； The double fluorescent report gene detection kit was bought from Promega company； The antibodies used for Western blot are purchased from Santa Cruz. Xho1 and BamH1 enzymes are purchased from Takara Biology; PCR kit is purchased in Thermo Fisher; Plasmid Extraction Kit and purification kit purchased from Tiangen Biotechnology (Beijing) Co., Ltd..

2.2 Bioinformatic analyses

The software miRBase (http://mirbase.org/) and Targetscan (http://www.targetscan.org/) were used to identify and analyze potential microRNA target sequences involved in the regulation of carp gene expression. The 3'-UTR ΔG was calculated with the use of the software mFOLD (http://frontend.bioinfo.rpi.edu/applications/mfold/)[16].

2.3 Plasmids construction

The 3'-untranslated region (3'-UTR) of mRNA sequence of carp containing predicted miR-1397 binding site was amplified by PCR following the protocol of Primer star kit (Takara), the corresponding mutant constructs were created by mutating the seed regions of the miR-1397-binding sites. The primers used in the reaction were listed in Table 1. PCR products were cloned into the pGL3 reporter vector (Promega, USA).

Table 1. Primer sequences for building a vector

	primer (5'→3')
pGL3-ANKRD1 3'-UTR	3'UTR-F(CACAACTCGAGCCCGGAGGAAAATAGCCCTG)
	3'UTR-R（AAGGATCCGCTTCAGGAACTGTGTGGGA)
pGL3-ANKRD1 3'-UTR mut	3'UTR-MR (AGATTCGTAACGAATGTAAACTAT)
	3'UTR-MF(ACATTCGTTACGAATCTTTGTCAGCAACATGAGCC)

2.4 CFM cells culture and transfection

CFMs were prepared from the pectoralis muscles of 10-day-old White Leghorn chicken embryos and cultured as described previously[9,10]. Chicken fibroblast cells were prepared as described by Mar et al [17]. And maintained in DMEM supplemented with 10% fetal bovine serum. Chicken fibroblast cells were maintained under 5% CO2 at 37°C in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were cultured to 70% confluence and transiently transfected using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions.

2.5 Luciferase reporter assay

For identification miR-1397 target chicken carp 3'-UTR, reporter plasmids (pGL3 reporter vector containing carp 3'-UTR) and miR-1397 mimics were cotransfected into 293T cells (60% confluence) using Lipofectamine 2000, control groups here include cells cotransfected with pGL3/carp 3'-UTR and miR-NC, pGL3/carp 3'-UTR mutant and miR-1397 mimics as well as pGL3/carp 3'-UTR mutant and miR-NC. After 48 h cells were lysed and reporter activity was determined using Dual-luciferase report assay system (Promega, USA). Firefly luciferase values were normalized to Renilla, and the ratio of firefly/renilla was presented.

2.6 Tissues collection

All experimental procedures were conducted in conformity with institutional guidelines for the care and use of laboratory animals, and protocols were approved by the Institutional Animal Care and Use Committee in Guangdong Medical University. Various tissues including brain, heart, liver, spleen,

lung, kidney, chest muscles, skeletal muscle, pancreas, stomach, ovary, oviduct, skin and fat were collected for RNA extraction.

2.7 Real time PCR and Westernblot analysis

Total RNA extraction and quantitative real time PCR was performed as previously described [10]. Primers used were as follow: GAPDH, Forward: 5'-CCTCTCTGGCAAAGTCCAAG-3'; Reverd: 5'-GGTCACGCTCCTGGAAGATA-3'; carp, Forward: 5'-CCACTGGCAAACATGATGAC-3'; Reverse: 5'-CTCGCCTCACGCTTCTTCT-3'. MiR-1397 and 5s RNA qPCR primers were purchased from Integrated Biotech Solutions. Fold changes were calculated using the Δ Δ Ct method. Three independent experiments were performed in triplicate. Protein expression in CFM was analyzed by western blot as previously described.

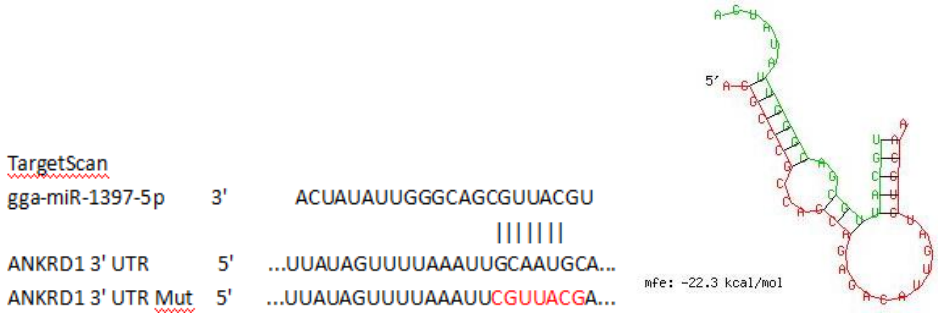
2.8 statistical analysis

Statistical analysis was analysed by Prism5 software. Data were presented as the means ± s.e. from at least three separate experiments. The two independent sample t-test was used to draw a comparison between groups. All tests were two-tailed, and the significance level was set at $P<0.05$.

3. Results

3.1 miR-1397 combine with carp 3′-UTR sequence

The chicken carp gene has a 3'-UTR that is more than twice as long as it's ORF, raising the possibility that it might contain post-transcriptional regulatory elements that would control its protein level. To search for miRNA (s) that might regulate chicken carp expression, we used the following target prediction algorithms: miRanda (http://microrna.sanger.ac.uk) and Targets can (http://www.targetscan.org). These approaches are based on the identification of elements in the 3'-UTR of target genes complementary to the seed sequence of the miRNA of interest, calculation of thermodynamic properties of 3'-UTRs. We found a miR-1397 putative binding site (GCAAUGCA) at the 3'-UTR sequence of carp (Fig.1A). This suggested that carp potentially was a regulator target for miR-1397. The seed match between miR-1397 and carp 3'-UTR is between bases 168 and 175 (Fig.1A). The free energy required for the interaction between miR-1397 and its cognate carp 3'-UTR binding site is -22.3 kcal/mol based on the Pictar prediction (Fig.1B).



A B

Figure 1. Prediction of miR-1397 target sequence in chicken carp-3 '-UTR.

(A) Schematic diagram of chicken carp mRNA containing the predicted conserved target site of miR-1397 and mutant (carp-3'-mUTR) miR-1397 target sequences. (B) Predicted hybridization of miR-1397 (green) and the chicken carp (red) transcript using the RNAhybrid algorithm [16]. The minimum free energy required for the hybridization is indicated.

3.2 miR-1397 directly targets chicken carp

To confirm chicken carp as a real miR-1397 target, the entire wild-type 3'-UTR of carp was inserted downstream of the firefly luciferase reporter gene and cotransfected with miR-1397 mimics into HEK293T cells. As shown in Fig.2A, co-transfection of miR-1397 mimics with the carp 3'UTR reporter resulted in a highly significant decrease (11.8 %, P=0.016) in luciferase activity. To confirm that the predicted target sequence of miR-1397 in the carp 3'-UTR is functional, this 7 bp seed region

was mutagenized as shown in Fig.1A. Notably, miR-1397 could not inhibit luciferase activity from the mutagenized carp 3'-UTR construct (Fig.2B), indicating that the predicted sequence is indeed a genuine binding site for miR-1397 and it is solely responsible for miR-1397 targeting of the carp 3'-UTR.

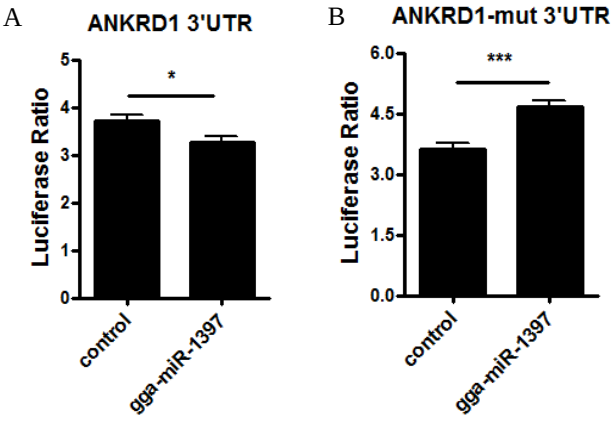


Figure 2. Target validation of miR-1397 using a luciferase assay system.

(A-B) carp 3'-UTR reporter vector and Nucleotides in the seed-binding region of the predicted binding site of miR-1397 in the 3'-UTR of the carp was mutated by site directed mutagenesis as depicted in Fig.1 and the reporter-construct was co-transfected with miR-1397 in H9C2 cells. After 24 h the cells were harvested and luciferase activity was measured. Data are presented as percental changes of relative light units (RLU) to control (miR-c) and indicated as mean±s.e. of six triplicates (n= 6; one-way ANOVA, *p< 0.05).

3.3 miR-1397 inhibits the expression of chicken carp

Furthermore, Western blotting analyses showed that carp protein levels were decreased after CFM cells received miR-1397. Consistently, endogenous level of carp protein was significantly increased in CFM cells (Fig.3A-B) by using this specific inhibitor against miR-1397. These results confirm that miR-1397 inhibits carp expression through the 3'-UTR, and further suggest that endogenous miR-1397 actively suppresses carp expression in CFM cells, which could be blocked by miR-1397 inhibitor.

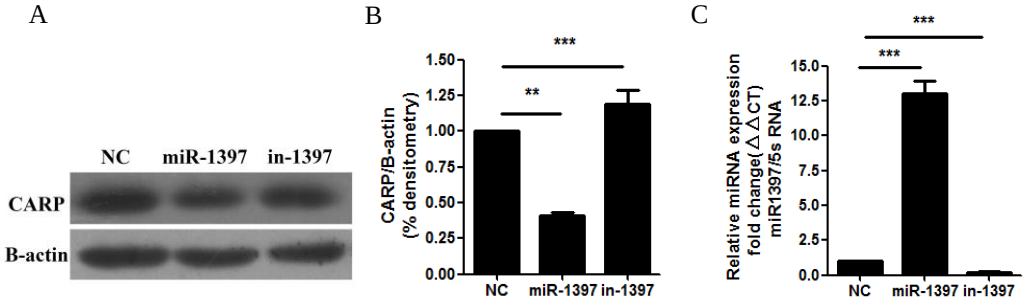


Figure 3. MiR-1397 directly reduces carp protein expression.

(A) CFM cells were transfected with the indicated concentrations of miR-1397 or anti-miR-1397 inhibitor (2'-Omethyl) or control (scrambled miR sequence) and harvested 48 h later. A representative Western blot using carp antibody is shown. (B) Mean amount of carp measured from three independent experiments was normalized to β-actin.The expression comparisons were significant with p< 0.001. (C) CFM cells express miR-1397. Representative bar shows the products of real-time PCR amplified with primers specific for miR-1397 in CFM cells transfected with scramble or mir-1397 or miR-1397 inhibitor. The amount of starting template for each condition was equilibrated relative to 5S rRNA. The expression values were normalized with 5S rRNA expression, and are represented as mean±s.e.(n=3, **p< 0.001).

3.4 Downregulation of miR-1397 during differentiation in CFMs is inversely correlated with the carp expression pattern

To gain insight into the function of carp, we examined its expression in adult chicken tissues via real-time PCR analysis. The results revealed that transcript of miR-1397 was widely expressed in all 12 tissues tested adult chicken tissues including skeletal muscle, liver and kideny, but especially predominantly expressed in brain and heart (Fig.4A). To characterize the expression pattern of miR-1397 in vitro, the CFM cells were seeded in 35 mm dishes at 4.0 x 104 cells per dish and harvested. As shown in Fig.4C, carp mRNA were increased, whereas the expression of mature miR-1397 was correspondingly reduced during CFMs differentiation (Fig.4B), suggesting that down-regulation of carp protein involves translational inhibition mechanisms, possibly dependent on the corresponding increased accumulation of miR-1397. Note that expression of carp is probably a combination of transcriptional and post-transcriptional mechanisms, in keeping with previous reports showing that inhibition of carp accumulation in is due to reduced transcription and to protein destabilization [8, 20].

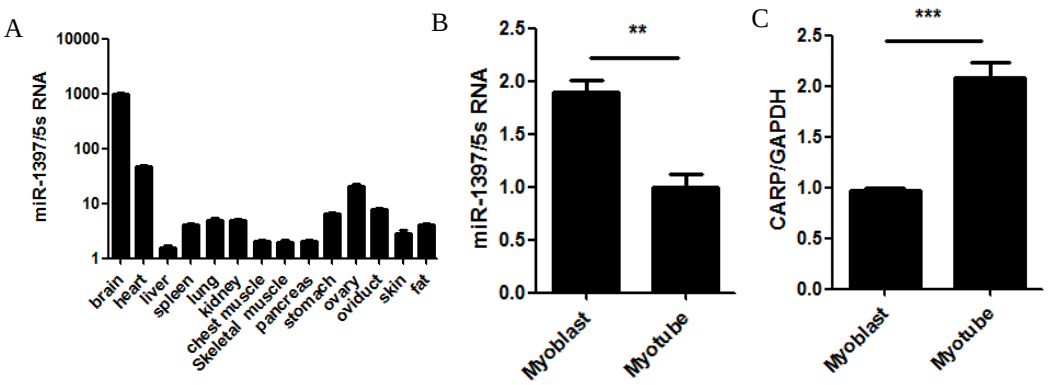


Figure 4 Expression of miR-1397 in chicken tissues and CFM cells.

(A) Expression of miR-1397 in adult chicken tissues. Real-time PCR show that miR-1397 expression is highly expressed in brain and heart. (B-C) miR-1397 expression during myogenesis. Total RNA enriched for miRNAs was extracted from chicken myoblasts and myotube (fused myoblasts in differentiation medium for 5 days), and analyzed by real-time PCR to assess miR-1397 and carp expression. The levels of miR-1397 decreased during myogenesis. On the contrary, the expression of carp increased. Representative bar shows the products of real-time PCR amplified with primers specific for miR-1397 and carp in chicken myoblasts and myotube cells. The amount of starting template for each condition was equilibrated relative to 5S rRNA or GAPDH. The expression values were normalized with 5S rRNA or GAPDH expression, and are represented as mean±s.e. (n=3, $p< 0.01$).

4. Discussion

The chicken carp gene has a 3'-UTR that is more than 2.2 kb [10], which indicated that controlling the promoter activity during transcription is not the only mechanism for carp regulation in muscle. MicroRNAs are known to negatively regulate the expression of their target mRNAs via the degradation of the bound mRNA target or by directly inhibiting translation of the target mRNA [18, 19]. However, while precise regulation of carp levels is an important determinant in defining the biological outcome, the role of miRNAs in modulating carp expression has not been extensively analyzed.

In this study, we demonstrated that carp is a direct target of miR-1397 in CFMs. Several lines of evidences support this conclusion. First, computational prediction of targets identified a putative binding site on the 3′-UTR of carp mRNA. This was experimentally validated by utilizing a luciferase reporter activity assay which showed that the miR-1397 mimic decreased the luciferase activity of the reporter vector containing the miR-1397 response elements; in contrast, the introduction of mutations

in the miR-1397 binding site of the carp 3'-UTR substantially reduced miR-1397's inhibitory effects. Second, miR-1397 induction correlated with a reduction in carp protein in CFMs. Third, inhibition of miR-1397 up-regulated, whereas the miR-1397 mimic down-regulated carp protein in CFMs. Together, these results demonstrate a functional significance of miR-1397 induction in inhibition of carp protein expression. However, we found no miR-1397 target sequences exist within the 3'-UTR of mammalian carp homolog genes, which may be correlated with homologous with chicken and mammalian carp is low and 3-'UTR sizes and sequences largely difference.

To our knowledge, miR-1397 is the first example of a chicken-specific regulator of carp levels. However, there are no published results on miR-1397 function in any animal or human model. Although our results indicated that miR-1397 is predominant expressed in brain and heart of the adult chicken, which relative lower expressed in skeletal muscle. Our study further contributes to this model and reports for the first time that miR-1397 expression levels are down-regulated during myogenesis. This coincides with the expression pattern of the chicken carp, which was predominantly expressed in chicken postnatal skeletal muscle, and its expression increased during myogenic differentiation in CFM cells [9, 10]. Our previous study demonstrated that carp may play an essential role in governing chicken skeletal myogenesis through promoting proliferation and avoiding apoptosis during CFM cell differentiation. Based on these results, we propose that down-regulated expression of miR-1397 is required during terminal differentiation in order that the positive regulator of myogenesis, carp, is rapidly and efficiently induced, thereby promoting myogenesis (Fig. 4).

In summary, our study demonstrated that the miR-1397 is an important factor affecting the carp gene expression in chicken. These data are helpful for elucidating the biological functions of carp and the molecular mechanisms underlying skeletal muscle development in avian. We provided a starting point for examination of the regulation of the chicken carp gene in more detail. Further work is required to elucidate the subtle molecular mechanisms controlling carp expression during myogenesis.

Acknowledgements

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References

[1] Chu W., Burns D.K., Swerlick R.A., et al. Identification and characterization of a novel cytokine-inducible nuclear protein from human endothelial cells. J. Biol. Chem. Vol. 270 (1995), 10236-45.

[2] Zou Y., Evans S., Chen J., et al. a cardiac ankyrin repeat protein, is downstream in the Nkx2-5 homeobox gene pathway. Development. Vol. 124 (1997), 793-804.

[3] Miller M.K., Bang M.L., Witt C.C., et al. The muscle ankyrin repeat proteins: CARP, ankrd2/Arpp and DARP as a family of titin filament-based stress response molecules. J. Mol. Biol. Vol. 333 (2003), 951-64.

[4] Zhang N., Xie X., Wang J. Multifunctional protein: cardiac ankyrin repeat protein. Journal of Zhejiang University. Science.Vol. B 17 (2016), 333-341.

[5] Zolk O., Frohme M., Maurer A., et al. Cardiac ankyrin repeat protein, a negative regulator of cardiac gene expression, is augmented in human heart failure. Biochem. Biophys. Res. Commun. Vol. 293 (2002), 1377-1382.

[6] Aihara Y., Kurabayashi M., Saito Y., et al. Cardiac ankyrin repeat protein is a novel marker of cardiac hypertrophy: role of M-CAT element within the promoter. Hypertension. Vol. 36 (2000), 48-53.

[7] Arimura T., Bos J., Sato A., et al. Cardiac Ankyrin Repeat Protein Gene (ANKRD1) Mutations in Hypertrophic Cardiomyopathy. Journal of the American College of Cardiology. Vol. 54 (2009), 334-342.
[8] Zolk O., Marx M., Jackel E., et al. Beta-adrenergic stimulation induces cardiac ankyrin repeat protein expression: involvement of protein kinase A and calmodulin-dependent kinase. Cardiovasc. Res. Vol. 59 (2003), 563-572.
[9] Yang W., Zhang Y., Ma G., et al. Identification of gene expression modifications in myostatin-stimulated myoblasts. Biochemical and Biophysical Research Communications. Vol. 326 (2005), 660-666.
[10] Ma G., Wang H., Gu X., et al. CARP, a myostatin-downregulated gene in CFM cells, is a novel essential positive regulator of myogenesis. International Journal of Biological Sciences. Vol. 10 (2014), 309-320.
[11] Lee S. Regulation on muscle mass by myostatin. Annu. Rev. Cell. Dev. Biol. Vol. 20 (2004), 61-86.
[12] Kambadur R., Sharma M., Smith T., et al. Mutations in myostatin (GDF8) in double-muscled Belgian Blue and Piedmontese cattle. Genome Research. Vol. 7 (1997), 910-916.
[13] Lee S. Extracellular Regulation of Myostatin: A Molecular Rheostat for Muscle Mass. Immunology, Endocrine & Metabolic Agents in Medicinal Chemistry. Vol. 10 (2010), 183-194.
[14] Cannell I., Kong Y., Bushell M.G., et al. How do microRNAs regulate gene expression ? Biochemical Society Transactions. Vol. 36 (2008), 1224-1231.
[15] Rao P., Kumar R., Farkhondeh M., et al. Myogenic factors that regulate expression of muscle-specific microRNAs. Proceedings of the National Academy of Sciences. Vol. 103 (2006), 8721-8726.
[16] Krüger J., Rehmsmeier M. RNAhybrid: MicroRNA target prediction easy, fast and flexible. Nucleic Acids Research. Vol. 34 (2006).
[17] Mar J., Antin P., Cooper T., et al. Analysis of the upstream regions governing expression of the chicken cardiac troponin T gene in embryonic cardiac and skeletal muscle cells. Journal of Cell Biology. Vol. 107 (1998), 573-585.
[18] Stark A., Brennecke J., Bushati N., et al. Animal microRNAs confer robustness to gene expression and have a significant impact on 3′UTR evolution. Cell. Vol. 123 (2005), 1133-1146.
[19] Callis T., Deng Z., Chen J., et al. Muscling Through the microRNA World. Experimental Biology and Medicine.Vol. 233 (2008), 131-138.
[20] Samaras, S. E., Chen, B., Koch, S. R., et al. 26S Proteasome regulation of Ankrd1/CARP in adult rat ventricular myocytes and human microvascular endothelial cells. Biochemical and Biophysical Research Communications. Vol. 425 (2012), 830-835.