Disruption of mstn Gene by CRISPR/Cas9 in Large Yellow Croaker (Larimichthys crocea)

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Short Report

Keywords: mstn, CRISPR/Cas9, large yellow croaker genome editing

Posted Date: April 14th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1524321/v1

Abstract

The large yellow croaker (*Larimichthys crocea*) plays an economically vital role in the marine aquaculture in China. Suffering from infection of bacteria and protozoon, effect of extreme weather and stress from high density farming, genome editing is thought to be an important tool applied to *L. croea* for enhancing commercial traits such as growth rate, disease resistance and nutrition component. In this study, we identified two *mstn* genes in *L. croea* and investigated the different phylogenetic clades, gene structures, and conserved syntenic relationships. To obtain fast-growing large yellow croaker, we specially selected two validated targets for *mstnb* knockout, which was homologous to mammalian myostatin gene (*MSTN*) and down-regulated skeletal muscle growth and development. Five significant mutation types were generated in two mosaic mutants by transferring specific CRISPR/Cas9 RNPs (ribonucleoprotein) into the one-cell fertilized embryos based on CRISPR/Cas9 technology. Subsequently, we also elucidated the obstacles and possible measures to improve the success rate of inducing modified large yellow croaker. Our results would provide valuable method and reference for facilitating genome editing programs of the large yellow croaker in the future.

Introduction

The large yellow croaker (*Larimichthys crocea*), which belongs to Perciformes, Sciaenidae and *Larmichthys*, possessing wonderful taste and tasty meat, is one of the most importantly economical marine fish species in China (Ke et al., 2022). Almost due to the breakthrough of artificial reproduction and the booming of aquacultural product, not only has it been the best seller among marine fish in China but also popular abroad (Zhao et al., 2021). However, there is a long breeding cycle from fry size to commercial fish size, and the large yellow croaker is susceptible to infection by pathogenic microorganisms during the high temperature period in summer, leading to the collapse of the industry (Bai et al., 2020). In recent years, researchers have devoted themselves to the studies on pathogens of large yellow croaker and breeding of stress-resistant families to decrease the annual loss of large yellow croaker industry (Bai et al., 2020, Zhao et al., 2021, Zhang et al., 2022). Compared with traditional long-period selection breeding, gene editing breeding provides a more effective and convenient promise with germplasm improvement, targeting the genes which regulate the economic traits.

Myostatin (*mstn*), also called growth and differentiation factor 8 (*gdf8*), is one of the members in the transforming growth factor beta (GDF-β) superfamily, mainly expressing a type of secreted glycoprotein in skeletal muscle (Kambadur R, 1997), which has a negative regulatory effect on the growth and development of skeletal muscle (Sun et al., 2020). When the *mstn* gene was mutated, the animals muscle mass would be greatly improved, which had been reported in mouse (*Mus musculus*) (Nassar et al., 2022), pig (*Sus scrofa*) (Qian et al., 2022), cattle (*Bos taurus*) (Meyermans et al., 2022), zebrafish (*Danio rerio*) (Gao et al., 2016), common carp (*Cyprinus carpio*) (Shahi et al., 2022), and yellow catfish (*Pelteobagrus fulvidraco Richardson*) (Zhang et al., 2020b), resulting in proliferation of myofiber size and cell number. The *mstn* gene has a conserved genetic structure with 3 exons and 2 introns in different species including mammals and teleost. Whereas, different to mammals with only one *MSTN* gene, *mstna* and *mstnb* were identified in bony fish, such as zebrafish (Kerr T, 2005), channel catfish (*Ictalurus punctatu*) (Zhang et al., 2020a), and blunt snout bream (*Megalobrama amblycephala*) (Sun et al., 2020). It was thought to be the result of fish-specific genome duplication (FSGD or 3R) occurred approximately 350 Mya ago (Christoffels et al., 2004). In 2015, the *mstnb* gene of large yellow croaker was located on chromosome 1 (Ao et al., 2015). But another *mstn* gene, the metabolism mechanism and phylogenetic investigation of *mstn* in *L. croea* were rarely reported, also including the reverse genetic work.

CRISPR/Cas9 system is an adaptive immune defense derived from bacteria through the long-term evolution to fight against invading viral integration, which consists of Cas9 protein and artificially engineered chemical RNA (Single guide RNA, sgRNA). Under the guidance of sgRNA after recognizing PAM (protospacer adjacent motif, PAM) sequence before the target, Cas9 protein would cut the specific sequences to generate DNA double strand break (DSB). After that, target gene knockout could be achieved by using the mechanism of Non-Homologous End Joining (NHEJ) without repair template (Hruscha et al., 2013). But CRISPR/Cas9 was not a first-generation gene editing technology. In 1987, scientists established a gene knockout model of mouse embryonic stem cells, and then the first generation of gene editing technology, zinc-finger nucleases (Zinc-finger nucleases, ZFN) technology and the second generation of gene editing technology, Transcription activator like effector nucleases (TALEN) technology rapidly developed and applied (Bibikova M, 2002, Kim and Kim, 2014). Moreover, gene knockout technology in aquaculture was achieved for the first time with the *mstna*-knockout yellow catfish gained by ZFN in 2011 (Dong et al., 2011). Insufficiently, the transformation of the DNA-binding domains of these two technologies was relatively complicated, especially for ZFNs, which often required the construction of a huge zinc finger expression library, and the screening workload was large, which greatly impeded its development (Klug, 2010). Hence its accurate recognition and convenient operation, CRISPR/Cas9 technology has been implied to several economically crucial aquaculture fish species, for gene function exploration, disease resistance research and genetic breeding. Especially, the improving induction of the red sea bream with an increase of skeletal muscle mass (Kishimoto et al., 2018), muscle mass enhancement in the olive flounder (*Paralichthys olivaceus*) (Kim et al., 2019), and the effective disruption of reproductive competence in Nile tilapia (*Oreochromis niloticus*) (Li et al., 2020) were accomplished by CRISPR/Cas9.

In this study, to explore the application of gene editing in increasing the yield of *L. croea*, we identified and classified *mstn* genes in *L. croea*, then designed gRNAs for knockout work of *mstnb*. It would be a pioneer to KO *mstn* in the large yellow croaker by microinjection based on CRISPR/Cas9 system, which could provide a significantly valuable case for future gene-editing breading work in the large yellow croaker.

Materials And Methods

Ethics statement

This study was approved by the Animal Care and Use committee at College of Ocean and Earth Sciences, Xiamen University. The methods were carried out in accordance with approved guidelines.

Identification of mstn **in large yellow croaker**

The genome data of the large yellow croaker was downloaded from the National Genomics Data Center (https://ngdc.cncb.ac.cn/gwh/Assembly/500/show). 8 MSTN/Mstn protein sequences of human (*Homo sapiens*), mouse, chicken (*Gallus gallus*), wall lizard (*Podarcis muralis*), zebrafish, and catfish were obtained from Uniprot website (https://www.uniprot.org/) (Supplementary Table S 2) They were used as queries to identified the potential Mstn in *L. croea* by blastp analysis with the mode of –ultra-sensitive and e-value set at 1e-5. Then the identified sequences were validated by the online tools SMART (http://smart.embl.de/) and CDD (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Only sequences contained TGF beta domain were retained to the following analysis. To classify the potential Mstns, 8 MSTN/Mstn protein sequences as reference and potential sequences were aligned together by ClustalW method and phylogenetic relationships were built with using the neighborhood-joining (NJ), 1000 bootstrap method and Poisson model by MEGA (v7.0.26) software (Kumar et al., 2016). Simultaneously, for further demonstrating the results of identification, chromosome location and synteny relationship between *mstn* and its neighborhood genes were graphically displayed. What's more, two *mstn* genes in *L. crocea*, *mstna* and *mstnb*, were obtained to analyze gene structure and extract exons sequences for targets design.

Design Of Target Sites And Primers

According to the exons sequences gained above, 9 sgRNAs (Table 1) were designed in total which targeted the 1st exon and 2st exon of *mstnb* gene in *L. crocea* by sgRNAcas9 software (Xie et al., 2014) and the specificity was verified by CasOT software (Xiao A, 2014). Refer to previous method (Gagnon et al., 2014), firstly, the gene-specific oligonucleotides which consist of T7 promotor (5'-GAA TTA ATA CGA CTC ACT ATA GG-3'), specific target sequence without the PAM (NGG) and complementary region (5'-GTT TAA GAG CTA TGC TGG-3') and the universal primer R were synthesized from Biosune Biotechnology Co., LTD. (Shanghai, China). Then the PCR reaction contained gRNA oligonucleotides and universal primer R were conducted to generate double-stranded templates for gRNA transcription. To synthesize the sgRNA, vitro transcription was performed under the RNase-free conditions by HiScribe T7 Quick High Yield RNA Synthesis Kit (NEB #E2040) and the purification was conducted with Monarch RNA Cleanup Kit (NEB #T2040L). The concentration and purity of sgRNAs were measured by Nanodrop spectrophotometer (Thermo scientific) and agarose gel electrophoresis using the Mini PROTEAN Tetra electrophoresis unit (Bio-Rad) respectively. The primers for fragment amplification spanning the target site were designed on the NCBI primer design website (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) and all oligos were submitted to Biosune Biotechnology Co., LTD. (Shanghai, China) for synthesis (Table 2).

Table 1
The primers for synthesis of *mstnb* gRNAs in *L. croea*.

Primers	Location	Target sequences (5'-3')
*mstnb*_gRNA1	exon1	GAATTAATACGACTCACTATAGGTCTTGGTCACTCAAAACTACGTTTAAGAGCTATGCTGG
*mstnb*_gRNA2	exon1	GAATTAATACGACTCACTATAGGACTATGTCTCGGCTGATATTGTTTAAGAGCTATGCTGG
*mstnb*_gRNA3	exon1	GAATTAATACGACTCACTATAGGCGTGCTGGGAGATGACAACAGTTTAAGAGCTATGCTGG
*mstnb*_gRNA4	exon2	GAATTAATACGACTCACTATAGGACTTTGGTTCCTCATCCACTGTTTAAGAGCTATGCTGG
*mstnb*_gRNA5	exon2	GAATTAATACGACTCACTATAGGGTCATTTCCCCTCGAATCGAGTTTAAGAGCTATGCTGG
*mstnb*_gRNA6	exon2	GAATTAATACGACTCACTATAGGGGCATTCACGTCGATCTTCAGTTTAAGAGCTATGCTGG
*mstnb*_gRNA7	exon2	GAATTAATACGACTCACTATAGGGAAGATCGACGTGAATGCCGGTTTAAGAGCTATGCTGG
*mstnb*_gRNA8	exon2	GAATTAATACGACTCACTATAGGACTTTGCCAAGAGCTGACCCGTTTAAGAGCTATGCTGG
*mstnb*_gRNA9	exon2	GAATTAATACGACTCACTATAGGGATTAACGCCTTCGATTCGAGTTTAAGAGCTATGCTGG
universal reverse		AAAGCACCGACTCGGTGCCACTTTTTCAAGTTGATAACGGACTAGCCTTATTTAAACTTGCTATGCTGTTTCCAGCATAGCTCTTAAAC
The underline sequences indicated the targets.		

Table 2
The primers for PCR amplification of target sites.

Primers	Sequences	Tm (°C)	Product length (bp)
*mstn*_gRNA1_3 F	TGTGAGGACTCGTTCAGCAC	60	550
*mstn*_gRNA1_3 R	GATAGCGTCTCCAATGCGCT	60	
*mstn*_gRNA4_9 F	ACCAGTGTATTGGTCGGTGC	60	550
*mstn*_gRNA4_9 R	AAGGTTCAGCTCACCAGTCC	60	

Artificial fertilization of L. crocea **and microinjection**

The mature adult large yellow croakers in our study were selected from a commercial breeding company, Ningde Fufa Fisheries CO., LTD. (Fujian, China). The parental fish were reared in indoor cement tank at the 24 ~ 25 ℃. With average height of about 1-1.5 kg, 10 females were injected with 3 μg luteinizing hormone releasing hormone A3 (LRH A3) dissolved in amino acid solution respectively, and the dosage of male reduced by half. The sperm and eggs were collected by gently extruding the belly (Fig. 1a). After mixing sufficiently, adding clean seawater to activate sperm for fertilization. Fertilized eggs would float on the water and were transferred into petri dishes for microinjection. The *mstn*-sgRNA1 and *mstn*-sgRNA2 were mixed with Cas9 Nuclease (NEB #M0386M)

with 1:1 volume ratio for 10-minute incubation at room temperature. To better observation, a little phenol red was added to the mixture and the optimized solution of sgRNA/Cas9 RNPs was delivered into the embryos by microinjection at a final dosage of 500 ng/μL, using microinjector Peneumatic PicoPump (PV820, USA).

Amplicon Sequencing Of Target Region In G0 Fish

After microinjection, wild-type and injected embryos were incubated and reared respectively in filtered seawater with continuous aeration at 22 ℃ until hatching. At 24 hpf, the genomic DNA was isolated from embryos by extraction solution and neutralization solution (Dongshengbio #9052). PCR amplification of targeted *mstn* gene was carried out with a system of 12.5 μL 2× Premix Taq (TaKaRa RR901), 8.5 μL RNase-free water, 1 μL primer F/R and 2 μL extracted DNA. The program was finished with the parameters showed in the Supplementary Table S 1. The PCR products were examined by 2% agarose gel electrophoresis using the Mini PROTEAN Tetra electrophoresis unit (Bio-Rad) and purified by Fast ® Gel DNA Extraction Mini kit (Vazyme, #DC301-01). To check if there was a mutation in the target region, the PCR amplification were cloned into the pMD^{TM}19-T vector (TaKaRa) and then transformed into *E.coli* DH5α competent cells for Sanger sequencing.

Result And Discussion

Characteristics of the target gene mstn **in large yellow croaker**

Myostatin (MSTN/Mstn) is a significant inhibiting factor for muscle growing not only in mammals but also teleosts. Especially, due to the fish-specific whole genome duplication, two *mstn* genes, *mastna* and *mstnb*, were found in bony fish like zebrafish, channel catfish and grass carp (*Ctenopharyngodon idellus*) (Xu et al., 2019). In our study, via genome-wide blastp with the reference sequences, we also identified two *mstn* genes in the large yellow croaker, which had never been mentioned in other studies. As the phylogenetic result showed (Fig. 2a), one was homologous with the Mstnb protein sequences of zebrafish and channel catfish, renamed Mstnb. Meanwhile the other one presented closer relationship and conserved synteny with the Mstna sequences, therefore renamed Mstna. The phylogenetic result supported the different paralogous clades of Mstn in large yellow croaker which was coincided to previous results.

As for chromosome position (Fig. 2b), *mstna* was located in chromosome 19 while *mstnb* was in chromosome 1. What's more, it was also found that *mstnb* kept a relative conserved neighborhood location with *hibsh*, *psm1* and *ormdl1* genes on the same chromosome, which was the same as the *mstn* genes in mammals (Fig. 2b). And the paralogous copy, *mstna* was always collinearly surrounded by *ftcd*, *stat1*a and *glsa* genes (Fig. 2b). Both of them contained 3 exons but the whole length of *mstna* was longer than that of *mastnb* (Fig. 1b). MSTN/Mstn in vertebrate species consisted of a single peptide concluding an N-terminal predomain and a C-terminal active domain (Xu et al., 2003). According to the motif components of MSTN/Mstn, 8 motifs were the common and conserved elements among vertebrates. However, it seemed that Mstnb in teleosts had evolved a different motif compared to mammals while Mstna lost two motifs compared with Mstnb (Fig. 2a). This could be the great importance of character for distinguishing the two branches.

Above all, different chromosome location, neighborhood genes, motif components and phylogenetic relationship suggested two *mstn* genes different roles and functions in growth and metabolism.

Mutation of mstn **in** L. crocea

Among numbers of species, it was clear that *mstnb* played a significant role in negatively regulating growth. *Mstnb*-deficient zebrafish and loach (*Misgurnus anguillicaudatus*) showed significantly increasing muscle mass with muscle fiber hyperplasia (Gao et al., 2016, Tao et al., 2021), and the body weight and body length of the *mstnab* $^{+/-}$ common carp were significantly enhanced (Shahi et al., 2022), which provided a promising direction in aquaculture output of economic fish. Therefore, we selected *mstnb* of the large yellow croaker as the target gene. After detecting, two sgRNAs from eight targets we designed to target the coding sequences in the exon1 were effective (Fig. 1b). Compared with sequence in wild type fish, five deletion mutation were detected including 12-bp, 28-bp, 36-bp, 83-bp and 97-bp deletion simultaneously (Fig. 2c). It was likely that different from single gRNA microinjection (Shahi et al., 2022, Tao et al., 2021, Zhang et al., 2020b), the injection of multiple gRNAs simultaneously could induce larger segments deletion between two target sites (Fig. 2c). This method was also considered and employed a faster and effective strategy based on CRISPR/Cas9 system in the zebrafish retinal diseases model research (Unal Eroglu et al., 2018), helping efficiently influence the gene function with designing 162 target sites for 83 gene to establish a high-throughput mutagenesis pipeline (Varshney et al., 2015). However, in the previous study of single *MSTN*-gRNA knockout in the olive flounder, only up to 11-bp deletions or insertions were observed (Kim et al., 2019). Similar result was also presented in blunt snout bream with three targets for *mstna* and *mstnb* deficiency (Sun et al., 2020). In our study, we harvested two validated gRNAs for the 125-bp target sequences in exon1 and transferred them into fertilized embryos concurrently. Surprisingly we received up-to-90-bp deletion between the two targets, which provided useful information and experiment guidance for next-step gene-editing work in the large yellow croaker.

Up to now, microinjecting CRISPR/Cas9 system into one-cell embryos has been thought to be a pretty popular and convenient method to knockout or knock-in genes in bony fish, which was also ever conducted in the large yellow croaker (Li et al., 2021). What couldn't be ignored were the low hatching rate caused by damage after injection and low surviving rate due to the special character of clustering. Front-line production experience and observation studies told us it was impossible for a few large yellow croakers to grow up to maturity alone. Unlike other fish, it was said that they preferred to live in a group with a group size up to ten thousand. Even though microinjection could transfer the RNPs into the embryos successfully, most of eggs were hard to repair the tinny hole caused by needle tip to die after injection at a surviving rate of 1–2%, which was showed in this study. Therefore, it's still a huge challenge to obtained at least one thousand live injected eggs, not to mention one thousand live fries. Recently electroporation was reused to import plasmid or DNA into the germ cells of the channel catfish (Dunham et al., 2018) and zebrafish (Daneluz et al., 2020) before fertilization, which could conduct thousands of cells in a few minutes. Maybe it would provide a new direction to transfer gRNAs into the sperm cells or one-cell embryos of *L. croea*.

On the other hand, the existence of mosaic mutation was another obstructive factor to obtain mutative homozygote and extremely common in most gene-editing animals, which also appeared in this study. It was reported that the developmental pace of embryos when injection conducted and the injection volumes of oligonucleotide donor template were the main reasons that resulted in mosaicism (Straume et al., 2021, Jin et al., 2020). After two-cell development, the RNPs existed in different cells would prefer to induce different disruption at the target site randomly. Usually, series of further hybridization was considered to obtain homozygous mutants. The generation of mosaic individuals was a disadvantage for genetic F0 mutants, which also implied the necessity of finding more high-efficiency substitute for the import of RNPs.

Current Problems, Challenges And Future Perspectives

Through long-period cultivation and trials, the artificial breeding of *L. crocea* was overcame (Zhao et al., 2021). But with the increasing consumption and product need, germplasm was urged to be improved under the threat of diseases bursting frequently (Bai et al., 2022). Genomic editing technology had been applied to kinds of farm animals including pig, sheep, bream and carp to produce superior traits, which showed great prospect for optimizing genotypes of *L. crocea*. However, the application of CRISPR/Cas9 in aquaculture was still at early phase of development compared to its use in biomedical research. Whether the design and selection of targets, or the difficulty of microinjection, or breading challenge for a small number of fries posed a huge obstacle to gene-editing work of the large yellow croaker. With the fast upgrading and improvement of gene editing, solutions would be worked out and applied sooner or later. Taking into consideration food safety and ecological risks, related assessment and management also should be appropriately established and completed as soon as possible. Encouragingly, AquaAdvantage salmon (Waltz, 2017), Galsafe pig and GE Nile tilapia were approved for production recently with their significant target traits (Blix et al., 2021), which set good examples. As shown as the results in this study, the biggest problem was how to gently and effectively transfer the RNPs into the one-cell embryos at a large amount in a few minutes. Other methods like electroporation are worth trying and researching. All in all, there is still a long and tortuous road to explore for realizing genetic improving in the large yellow croaker.

Conclusion

The widely application and booming development of genome editing technology benefit from the sequencing technology and biological information. In our study, two *mstn* genes were successfully identified in *L. crocea*, renamed *mstna* and *mstnb*. The characters of phylogenic relationship, gene structure and protein domains were analyzed. Based on CRISPR/Cas9 system, effective sgRNAs were designed, synthesized and injected into embryos, inducing a large-scale deletion on the target sequence of the 1st exon in *mstnb*. Our research elucidated the operability of gene editing in *L. croea* by microinjecting gRNAs and Cas9 protein into embryos, providing valuable method and reference to facilitate genome editing program of the large yellow croaker in the future.

Declarations

Acknowledgements

Thanks for the help from Ningde Fufa Fisheries CO., LTD. (Fujian, China).

Funding

This study was supported by the National Natural Science Foundation of China (U21A20264); the Special Foundation for Major Research Program of Fujian Province (2020NZ08003); the Open Research Fund Project of State Key Laboratory of Large Yellow Croaker Breeding (LYC2021RS02).

Authors' contributions

PX and TZ conceived and supervised the study. MY and BL designed experiments including selecting target gene, targets design and gRNA synthesis, and wrote the manuscript. MY and BL performed the analysis and designed the charts and tables. JW and QK conducted the artificial insemination, then MY, BL and YB conducted the microinjection, and mutation detection was finished by BL. All authors have read and approved the manuscript.

Conflict of interest

The authors declare that no conflict of interests exists among them.

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Figures

Figure 1

The workflow of *mstnb* knockout in *L. croea* using CRISPR/Cas9 technology and the gene structure of *mstna* and *mstnb* in *L. croea* and effective targets in exon1 of *mstnb*. **a)** The three phares conclude gRNA design and synthesis, CRISPR experiment and mutation detection. **b)** Target sequences were in red and PAM sequence were added yellow bottom color.

Figure 2

Identification of *mstn* in *L. croacea*. **a)** The phylogenetic analysis and motifs analysis of MSTN/Mstn protein sequences; the unrooted tree was generated with MSTN protein sequences using the maximum-likelihood method by MEGA 7; the reliability was measured using 1000 bootstrap replicates. **b)** The synteny analysis of *mstn* genes in *L. croea* with that from Human, mouse, chicken, zebrafish, and channel catfish; the information of genes was obtained from NCBI website and *mstn* genes among all species were highlight. **c)** The mutation types of *mstnb*$^{+/-}$ in F0 mosaic of *L. croea*; PAM sequence and target sequence of wild type (WT) were in blue; target sequence position of all samples were colored by orange; the red "-" indicated deletion; and the number of indel variants was shown on the right of each clone.

Supplementary Files

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