Sexually Dimorphic Expression and Special Regulatory Sequence of *Dnali1* in the Olive Flounder *Paralichthys Olivaceus*

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Abstract

Dynein axonemal light intermediate chain 1 (*dnali1*) is an important part of axonemal dyneins and plays an important role in the growth and development of animals. However, there is little information about *dnali1* in fish. Herein, we cloned *dnali1* gene from the genome of olive flounder (*Paralichthys olivaceus*), a commercially important maricultured fish in China, Japan, and Korea, and analyzed its expression patterns in different gender fish. The DNA sequence of the entire gene contained a 771 bp open reading frame (ORF), two different sizes of 5' untranslated region (5'UTR), and a 1499 bp 3' untranslated region (3'UTR). Two duplicated 922 nt fragments were found in *dnali1* mRNA. The first fragment contained the downstream coding region and the front portion of 3'UTR, and the second fragment was entirely located in 3'UTR. Multiple alignments indicated that the flounder Dnali1 protein contained the putative conserved domain. Its expression showed sexually dimorphic with predominant expression in the flounder testis, and lower expression in other tissues. The gene with the larger 5'UTR was specifically expressed in the testis. The highest expression level in the testis was detected at stages Ⅱ and Ⅲ. Transient expression analysis showed that the 922 bp repeated sequence 3'UTR of *dnali1* down-regulated the expression of GFP at the early stage in zebrafish. The results implied that *dnali1* might play an important role in flounder testis, especially in the period of spermatogenesis, and the 5'UTR and the repetitive sequences in 3'UTR might contain some special regulatory elements for the cilia.

Introduction

Dyneins, a family of major cytoskeletal motors [1], have wide variety of cellular functions [2] and can be divided into two types, axonemal and cytoplasmic dyneins [3]. They consist of heavy, intermediate, light intermediate, and light chains with different molecular weight and function [4]. The roles of different axonemal dyneins and their assembly processes remain elusive in vertebrates including fish [5]. Dyneins are mainly components of cilium, which have been found in most, if not all, vertebrate organs. Prominent cilia form into sensory structures, the eye, ear, and nose. Cilia are also involved in developmental processes, including left-right asymmetry formation, limb morphogenesis, and the patterning of neurons in the neural tube [6]. Dynein light and intermediate chains are required for the motility of dynein heavy chain and the assembly of axonemal dynein into cilia and flagella [3, 7]. Dynein axonemal light and intermediate chain1 gene (*dnali1*) encodes one light-intermediate chain and contains a coiled-coil domain in C-terminal region. Dnali1 has been shown to form complexes with the dynein heavy chains, actin, and caltractin/centrin molecules [8]. In Human (*Homo sapiens*), *DNALI1* could be a candidate gene for patients suffering from the immotile cilia syndrome [9]. Splice-site mutations of green alga (*Chlamydomonas reinhardtii*) *dnali1* impaired the flagellar motility [8]. However, there is little information about *dnali1* in fish. Only a study in zebrafish (*Danio rerio*) showed that axonemal protein components *dnah5* and *dnali1* were absent in its primary ciliary dyskinesia with *mynd10* mutation [10].

Repetitive sequences are prevalent in genome and have been confirmed to play an important role in biological evolution by regulating gene function [11]. There are about 10 % of all genes containing tandemly duplicated exons in human genome [12]. For example, a duplicated exon of human *glycine*

receptor a-2 has been noted as a candidate for alternative splicing [12]. Similarly, repetitive sequences in 3' untranslated region (3'UTR) are also important because 3'UTR of an mRNA is essential for many biological activities such as mRNA stability, protein translation, sub-cellular localization, protein binding, and translation efficiency [13]. However, there is almost no relevant report for repetitive sequences of 3'UTR in vertebrates although the studies on function of 3'UTR have been performed. In murine spermatids and mammalian germ cells, transcription and translation of *Prm1* were controlled by a conserved element in 3'UTR [14, 15]. A miR430 recognition binding sequence was found in 3'UTR of zebrafish *nanos3* which could accelerate target mRNA decay after binding [16, 17], and the combined effect of codon usage and 3'UTR length determines the stability of maternal mRNAs in the embryos [18].

Olive flounder (*Paralichthys olivaceus*) is one of the most commercially important cultured marine fish species in Korea, China, and Japan [19]. There are growth differences between female and male individuals, and then study on the flounder sex control and its molecular regulation is valuable. According to our transcriptome data [20], *dnali1* was highly expressed in the flounder testis. However, similar to other fish, the detailed data of *dnali1* in the flounder are unclear.

In the present study, we cloned the *dnali1* and found the repetitive sequences in its 3'UTR. The molecular characteristic expression patterns in the flounder tissues and gonads, and the regulation function of repetitive sequences in 3'UTR were studied. The results will provide basic data for further investigation of the flounder spermatogenesis process.

Materials And Methods

Fish and sample collection

The wild-type flounders for tissue (25–35 cm total length, TL) and gonadal development (12–47 cm TL) analyses were purchased from Nanshan market (Qingdao, China), temporarily reared in aerated seawater tank at the institute aquarium and fed with commercial particle food twice a day. Twelve tissues (ovary/testis, brain, heart, muscle, head kidney, kidney, intestine, spleen, liver, stomach, gill, and eye) and gonads at development stages I to V were respectively dissected from three male and three female flounders after anesthetization with tricaine methane sulfonate (MS-222, 50 mg/L, Sigma, USA). Parts of the gonads were fixed in Davidson's fixative for histological section stained with hematoxylin/eosin (HE), which would be used to identify the genders and gonadal development stages [21]. The rest parts of the gonads and other tissues for RNA isolation were frozen as soon as possible and stored in -80 °C.

Zebrafish (TU strain) were reared in a recirculation culture system at the institute aquarium (temperature $28.5 \pm 1^\circ\text{C}$, photoperiod 14 h light : 10 h dark). Fish were fed with a commercial particle food twice and brine shrimp once every day. Fertilized eggs were obtained by mixing one male with two female fish in the morning. After washed with the cycling water several times, the eggs were ready for microinjection.

Total RNA extraction and cDNA synthesis

Total RNA was isolated from the flounder tissue and gonadal samples by using Trizol reagent (TOYOBO, Japan) following the manufacture's protocol. The quantity and purity of the RNA were assessed with electrophoresis in 1 % agarose gel and Nanodrop2000 (Thermo scientific, USA). After DNase I (Thermo scientific, USA) treatment, 1 µg total RNA was used for cDNA synthesis with M-MLV reverse transcriptase kit (Promega, USA). The obtained cDNA was preserved at – 20 °C.

Isolation of *dnali1* cDNA

Based on the flounder testis and ovary transcriptomic data [20] and the flounder genomic data (Genebank accession no. XM_020093361.1), the flounder *dnali1* sequence was cloned and verified. The ORF was cloned through semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) using the flounder testis or ovary cDNA as template and specific primers (*dnali1*-oF/oR, Table 1).

Table 1
Primers used in this study

Gene	Sequences (5'-3')	Function
<i>dnali1</i> -oF	ATGAGCCCACCAACAGATTCTCTCC	Gene clone
<i>dnali1</i> -oR	TCAGCTTTTCTTCGGTGTGGCG	Gene clone
3' gsp1	GCCCTCAGCAGCCCATTGACGCCTGCC	3'Race
3'gsp2	CCACCTCCACTGAGGCCACCGGGC	3'Race
5' gsp1	GCCCGGTGGCCTCAGTGGAGGTGG	5'Race
5' gsp2	GGGCAGGCGTCAATGGGCTGCTGAGGC	5'Race
genomic part A -F	TGCCTTTAATCCATATCTTTAACCC	Genomic clone
genomic part A-R	TGGGAAAAAGAAAAATCGGAG	Genomic clone
genomic part B-F	CTCTACGACCAGTGCTTT	Genomic clone
genomic part B-R	CCAATTGGATATTTTAATGA	Genomic clone
<i>dnali1</i> -RT-R	GGAGTGCTGGACACTTGC	RT-PCR
<i>dnali1</i> -RT-F	TCAGCTTTTCTTCGGTGTGGCG	RT-PCR
<i>dnali1</i> -5'utr-F	ACATGGGGATGGTATCATTACTCC	RT-PCR
<i>β-actin</i> -RT-F	GGAATCCACGAGACCACCTACA	qPCR/RT-PCR
<i>β-actin</i> -RT-R	TTGCTGATCCACATCTGCTG	qPCR/RT-PCR
<i>dnali1</i> -qF	TTCAATCACTTCCCAAAC	qPCR
<i>dnali1</i> -qR	TCCCTGCGAATGGGACA	qPCR
3'UTR-fragment 1/1-F	GGATGAACTATACAAATAGGGATC CAAACCTAAAAACCCTGAGTTACC	Vector construction
3'UTR-fragment 1-F	GGATGAACTATACAAAGGATCCGATGAGCT CATCCGACAGGTGACC	Vector construction
3'UTR-fragment 1/1/1-R	GGGAGCTCGCCCGGGGATCCCCAATTGA TATTTTAAT	Vector construction

The PCR was carried out in a mixture containing 2 μ L of cDNA (50 ng/ μ L) from the flounder testis or ovary, 12.5 μ L of 2 $\times$ GoldStar MasterMix (CW BIO, China), 1 μ L of forward primer (10 mM), 1 μ L of reverse primer (10 mM), and 8.5 μ L of RNase-free water. PCR was performed as follows: 95 $^{\circ}$ C for 10 min, 35 cycles of 95 $^{\circ}$ C for 30 s, 55 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min, and followed by a final extension at 72 $^{\circ}$ C for 10 min.

Isolation of 3'UTR and 5'UTR of the flounder *dnali1*

To obtain 3'UTR and 5'UTR sequences of the flounder *dnali1*, rapid amplification of cDNA ends (RACE) was performed with specific primers (3'gsp1 and 3'gsp2, and 5'gsp1 and 5'gsp2, Table 1) using SMARTer™ RACE cDNA Amplification Kit (Clontech, USA) according to the manufacturer's protocol. All the PCR products were purified using E.Z.N.A.® Gel Extraction Kit (Omega Bio-Tek, USA). The target fragments were cloned into pEASY-T3 vector (Transgen, China) and sequenced.

Isolation of genomic sequence of the flounder *dnali1*

The *dnali1* genomic sequence was cloned according to genomic sequences provided by Profs. Songlin Chen and Changwei Shao from Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. In order to confirm the repeated sequences in *dnali1* genomic sequences, specific primers (genomic part A –F/R, genomic part B –F/R, Table 1) were designed to clone the genomic sequences from three flounder fish (25–35 cm TL). The PCR was carried out in a mixture containing 2 µL of genomic DNA (50 ng/µL) from the flounder fins, 12.5 µL of 2 × KOD One™ PCR Master Mix (TOYOBO, Japan), 0.75 µL of forward and reverse primers (10 mM), and 9 µL of Dnase/RNase-free water. PCR was performed as follows: 98 °C for 10 min, 35 cycles of 98 °C for 10 s, 53 °C for 5 s, and 68 °C for 20 s, and followed by a final extension at 68 °C for 5 min. The PCR product was purified and ligated with TOPO-A clone and sequencing.

Transcription factor binding sites prediction

The putative specific transcription factor binding sites in 5'UTR were predicted by online tools program JASPAR (http://jaspar.binf.ku.dk/cgi-bin/jaspar_db.pl) using the JASPAR CORE databases [22].

Multiple alignments and phylogenetic tree construction

Multiple alignments were performed using DNAMAN. The evolutionary history was inferred by using the neighbor-joining method. Evolutionary analyses were conducted using MEGA7 [9].

RT-PCR

RT-PCR was performed with specific primers (*dnali1*-5'utr-F and *dnali1*-oR, Table 1) to test the differential expression of the alternatively spliced 5'UTR of *dnali1* in the flounder testis and ovary at stage Ⅱ.

The expression patterns of *dnali1* in the tissues of the adult flounders were evaluated using RT-PCR with specific primers (*dnali1*-RT-F/R, Table 1). *β-actin* (*β-actin*-RT-F/R, Table 1) was selected as a reference gene. PCR reaction mixture was exactly the same as described in the “*Dnali1* cDNA and 3'UTR cloning” and the annealing temperatures were 55 °C for *dnali1* and 62 °C for *β-actin*, respectively.

Quantitative expression analysis

The expression of *dnali1* in the gonads at stages Ⅱ - Ⅳ was analyzed using real-time quantitative polymerase chain reaction (qPCR). The templates were prepared using PrimeScript™ RT reagent Kit containing gDNA Eraser (Takara, Japan) with 1 µg total RNA. According to the manufacturer's instructions, the qPCR was performed using TB Green™ Premix Ex Taq™ II (Tli RNase H Plus) (Takara, Japan) in a 20 µL mixture containing 10 µL of 2×TB Green Premix Ex Taq II, 0.4 µL of ROX Reference Dye II, 0.4 µL of each forward and reverse primers (*dnali1*-RT-F/R, Table 1), and 1 µL of cDNA (50 ng/µL), on an Applied Biosystems QuantStudio6 qPCR machine (Life technologies, USA). The PCR was performed as follows, denaturation at 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s, 60 °C for 30 s, and then measured the melting curves. Reaction without template was used as negative controls. *β-actin* gene was used as a reference gene (*β-actin*-RT-F/R, Table 1) in the same condition [23]. Samples were run in triplicates and relative gene expression levels were calculated with the $2^{-\Delta\Delta Ct}$ method [24].

Plasmid constructions and microinjection in zebrafish embryos

According to the sequencing results, two 922 bp repeated sequence were found in *dnali1* mRNA. To investigate the regulatory activity of the flounder *dnali1* 3'UTR, different sizes of 3'UTR were cloned into pSP64-GFP vector [25]. Therein, fragment Ⅰ represented part sequences behind termination codon of the first repeat segment, fragment Ⅱ contained the second repeat segment, and fragment Ⅲ represented whole 3'UTR (fragments Ⅰ and Ⅱ). Three kinds of fragments were subcloned into the *Bam*HI site that located downstream of GFP coding region in pSP64-GFP vector. The regulating plasmids were constructed using homologous recombination method with SoSoo Cloning kit (TSINGKE, China) and specific primers (3'UTR-fragment Ⅰ/Ⅱ/Ⅲ-F/R, Table 1).

Chimeric RNA composed of GFP coding region and *dnali1* 3'UTR synthesized by *in vitro* transcription using a Message Machine SP6 Kit (Ambion, Thermo Fisher Scientific, USA). After purification, the concentration of the capped RNA was diluted to 300 ng/µL in 0.2 M KCl with phenol red (0.01 %). This solution (approximately 2 nL) was microinjected into the fertilized eggs of zebrafish at one-cell or two-cell stage. The injected eggs were cultured at 28.5 ± 1 °C. The GFP expression was observed under a fluorescence microscope (Nikon, Japan) at 72 h post fertilization (hpf).

Data analysis

One-way analysis of variance with Duncan post hoc test in SPSS 16.0 was used to test significant differences of gene expression among the tissues and gonadal development stages. Student's *t*-test was used to characterize significant differences between the testis and ovary at the same gonadal development stage. The threshold for significance was set as $P < 0.05$.

Result

Characterization of the flounder *dnali1* gene

Genomic sequence for coding region of the flounder *dnali1* contained 8 exons and 7 introns (Fig. 1a), and the exon/intron splicing positions were consistent with the GT...AG rule. Coding region was 771 bp, which encoded 256 aa. 3'UTR was 1499 bp long (Fig. 1a). There were two alternatively spliced variants in the first exon in 5'UTR (Fig. 1b). It is interesting to find that part of 3'UTR sequence coincided with partial sequence within ORF (Fig. 1). The deduced flounder *dnali1* amino acids shared 73%, 98%, and 61% identities with those of *Lates calcarifer*, *Scophthalmus maximus*, and *Cynoglossus semilaevis*. There was a putative conserved axonemal dynein light superfamily domain (pfam10211) in the flounder *dnali1* (Fig. 1c). C-terminal region of Dnali1 was more highly conserved, too. Phylogenetic tree showed that the flounder *dnali1* was clustered with those from other fish species such as *S. maximus* and *L. calcarifer* (Fig. 1d).

Confirmation of the large repeated sequence in the genome

About 2.6 kb fragments of *dnali1* genome were respectively cloned from three different flounders (Fig. 2a) and the sequencing results showed that there were two 1229 bp repeat segments. Comparison of the cDNA sequences and the obtained genomic sequences confirmed that there were 1229 bp repeated DNA sequences in genomic sequences, which spanned five exons in ORF and 3'UTR regions of the gene (Fig. 2b).

Sexually dimorphic expression of alternatively spliced 5'UTR of the flounder *dnali1*

RT-PCR was performed to determine whether alternatively spliced 5'UTR of the flounder *dnali1* has male or female specific expression pattern. The results showed that the larger one was only detected in the flounder testis, while the smaller one was found in both the ovary and testis (Fig. 3a). The sequences presented that one SOX5 and one SOX10 binding sites were lost in the smaller one (Fig. 3b).

Tissue distribution of the flounder *dnali1*

According to the histological analysis, the developmental stages of the testis and ovary of the adult flounder for tissue distribution analysis were stages Ⅲ and Ⅳ, respectively. The RT-PCR results showed that *dnali1* was mainly expressed in the testis, while less in the ovary. There was also weak expression in some other tissues or organs such as the male and female brain, heart, eye, and liver (Fig. 4a).

Dnali1 expression at stages Ⅲ-Ⅳ of the gonads

Based on the results of the gonadal histological sections (Supplementary Fig. 1), the flounder *dnali1* expression at stages Ⅲ-Ⅳ of gonadal development was tested using qPCR (Fig. 4b). Its expression was extremely higher in the testis than in the ovary ($P < 0.01$) except for stage Ⅲ. The lowest expression of *dnali1* was detected in the testis at stage Ⅲ, and the expression significantly increased at stages Ⅲ and Ⅳ ($P < 0.05$), then it continually significantly increased at stages Ⅲ to Ⅳ ($P < 0.05$). There was no significant difference between stages Ⅲ and Ⅳ, and Ⅲ and Ⅳ. The expression levels at stages Ⅲ and Ⅳ were approximately 90,000 times of that at stage Ⅲ. The expression in the ovary was low, and the lowest expression was presented at stages Ⅲ, Ⅳ, and Ⅳ.

Regulation activity of 3'UTR

Intriguingly, there were two 922 bp tandem repeated sequences in 3'UTR of *dnali1*. The first one contained the downstream coding region and front portion of 3'UTR, while the second one was entirely located in 3'UTR. To investigate contribution of the repeated sequence in 3'UTR to the stability and regulation activity of *dnali1* mRNA, GFP reporter plasmids containing different sizes of 3'UTR were constructed (Fig. 5a, b) and injected into zebrafish embryos. After hatching, the larvae were analyzed under a fluorescence microscope (Fig. 5c). GFP expression in the fragment α injected group (54/54) was the same as that of the control (59/59). While, there were about 65.91 % (29/44) and 52.08 % (25/48) of zebrafish injected with the fragments β and γ chimeric RNA showing strong GFP expression within heart area, respectively. The expression in the fragment β injected group was stronger than that in the fragment γ injected one.

Discussion

Herein, we isolated and characterized *dnali1* gene from the flounder. Its expression in adult tissues and the gonads at stages III – IV was analyzed. Expression of the flounder *dnali1* showed sexually dimorphic and its 3'UTR presented specific RNA retention function.

The flounder *dnali1* cloned in this study showed a putative dynein light intermediate chain gene with two splice isoforms of 5'UTR (5'UTR-S, 189 bp and 5'UTR-L, 419 bp). It is interesting to find that expression of the gene with the larger isoform was male specific. It was estimated that transcripts from 12 % of genes are alternatively spliced within 5'UTRs [26], and these variations in 5'UTR can function as important switches to regulate gene expression [27, 28]. Transcription factor binding sites and structural motifs in 5'UTR were predicted to analyze the different expression patterns in the male and female flounders. Comparison of the transcriptional factors binding sites, we found that one SOX5 and one SOX10 binding sites were absent in the smaller 5'UTR (5'UTR-S). SOX5 is a transcription factor with homology to the high mobility group box region of the testis-determining factor, SRY. Both of mouse and human SOX5 proteins were only present in tissues containing cells with motile cilia/flagella [29]. SOX10 was expressed in male-specific Sertoli cells only after sex determination in mouse, and required for the maintenance of male fertility in mammal [30]. Expression of *dnali1* was very high at stages III to IV in the testis, which suggested that the missed factors of 5'UTR-S, SOX5, and/or SOX10 in the ovary, may be key elements for the regulation of *dnali1* expression in the testis. Further study on difference between these two isoforms in regulating activity needs to be performed in the future.

Two sequences of 922 bp in the flounder *dnali1* gene were found, which stretched across its ORF region and 3'UTR region. In the genome, repetitive genomic DNA sequences were furtherly confirmed according to the results from three flounder individuals. This phenomenon hasn't been reported in vertebrates so far. To confirm this, *dnali1* 3'UTR sequences of amphioxus (*Branchiostoa floridae*) and two flatfish (*S. maximus* and *C. semilaevis*) were also cloned, sequenced, and blasted, and none of them has repeat sequences (data not shown). Repetitive sequences are prevalent in vertebrate genomes but they are

usually no more than 100 bp [31]. However, in tick-borne flaviviruses (TBFV), longer repeat sequences (about 200 bp) were found both in 3'UTR and ORF of the genome, which was supposed that 3'UTR might have overlapped function with ORF during the evolution of these viruses [31]. It was implied that multiple duplication of ORF terminal region might be the major event that shaped evolution of the TBFV genome. Tandem gene duplication is usually one of the most prevalent ways of generating genes with new function. Segmental duplication (tandem duplication of a genomic segment) contains both high-copy number repeat and gene sequences with intron–exon structure which occurs frequently to generate redundant genes during evolution [32]. But a minority segmental duplication only produces duplicated exons, rather than entire gene, which is called as tandem exon duplication and is an important source of new exons [33]. The tandem exon duplication is an important mechanism for expanding gene function [11, 34]. However, wrong tandem exon duplication might also cause negative effect. For example, in human MTM1 mutant genomic DNA, the duplicated MTM1 exon10 produced a 186 base-pair insertion in the MTM1 transcript, which demonstrated there was a necessary intronic sequence for recognition by the spliceosome [35]. The transcript containing the duplicated exon10 retained the reading frame of the wild type transcript and therefore a mutant polypeptide was generated. The repeat segment in the flounder *dnali1* is interesting, which duplicated partial genome including one intact exon (E4), one intact intron (I4), and a partial exon (partial E5). The first repeated exon (E4) fused with the second repeated exon (partial E5) to form the exon (E5) and the partial exon (partial E5) became new exon (E6). Besides, the first repeat segment included coding region and non-coding region which hasn't been reported in vertebrate before. So, this might be a new kind of evolutionary process. Further studies in more species should be performed to learn evolutionary role of the repeat sequences.

Dynein light intermediate chain protein is a component of the inner dynein arms (IDA), which is important for the assembling of axonemal dyneins [36]. Defects in the axoneme mostly in the structure of outer dynein arms (ODA) and IDA cause primary ciliary dyskinesia [37]. Axonemal abnormalities were observed in *C. reinhardtii* flagella if they lacked the *dnali1* product [36]. In human, DNALI1 might promote the stable assembly of the *dnali1*-containing dynein arms or their binding to the axoneme [38]. So, *dnali1* could be candidate gene for ciliary motility in mammals. In zebrafish, it has been proved that endothelial primary cilia play a pivotal role for its correct growth and development [39]. In medaka (*Oryzias latipes*), cilia integrity and axonemal localization of dynein arms were shown to be important for sperm dysmotility, scoliosis, and progressive polycystic kidney [40]. In the present study, *dnali1* was mainly expressed in the testis, while in the ovary, its expression kept a low level. The expression levels in the testis were different at different stages. A low level was detected at stage Ⅱ, and the highest level was shown at stages Ⅲ and Ⅳ, at which the secondary spermatocytes develop to spermatid quickly and the sperm flagella are formed [21]. This high expression results also supported role of *dnali1* at these flounder spermatogenesis stages. The sperm axonemal dynein motors consist of ODA and IDA [41]. Splice-site mutations in the gene encoding Dnali1 are correlated in the IDA-4 mutant with a loss of a set of IDA classes, indicating an important role of Dnali1 for the assembly of IDA isoforms [8]. In medaka, uncorrected axonemal localization of dynein arms caused sperm dysmotility [40]. These findings suggest that the *dnali1* gene plays an important role in fish spermatogenesis.

The heart is the first formed organ during embryogenesis [42]. In zebrafish, endocardial primary cilia of the heart, important mediators of fluid flow, play an important role in early embryonic development [43]. Although *dnali1* was less expressed in the flounder heart than in the testis, it was required for the motility of cilia and flagella [3, 7] and might have function in the flounder heart. It has been proved that 3'UTR of *dnali1* not only regulates mRNA-based processes, such as mRNA localization, mRNA stability, and translation, but also transmits genetic information encoded in 3'UTRs to proteins through the establishment of its mediated protein-protein interaction [44]. Zebrafish is an effective model fish to study gene function for its easily observation and microinjection, and has been used in the flounder successfully [45, 46]. Herein, we analyzed 3'UTR regulation activity of the flounder *dnali1* in zebrafish. The results showed that GFP expression in the fragment Ⅱ injected group (54/54) was the same as that in the control and fragments Ⅰ and Ⅲ injected groups, which could regulate GFP expression in heart and neural tube. The results suggested that the repeated code region (E4) might contain specific regulation elements and its deficiency would decrease mRNA stability, so it didn't show GFP expression in the fragment Ⅲ injected group. It was suggested that the repeated code region (E4) had dual function, which could control specific translation and maintain RNA stability. Recent study found that 5'UTR and ORF elements and 3'UTR, regulated the translation of Cyclin in mice [47]. In the flounder, 3'UTR might transmit genetic information encoded in 3'UTRs to proteins through the establishment of its mediated protein-protein interaction. Further studies should be performed to provide evidence to clarify the regulatory mechanism of the repeat sequences.

Conclusion

In this study, we isolated and characterized *dnali1* gene in the flounder. *Dnali1* was mainly expressed in the testis, and peaked at its stages Ⅱ and Ⅲ. However, the two repeated sequences across ORF and 3'UTR regions of the gene were only found in the flounder and they had different regulation activities. This is the first report about *dnali1* gene in marine fish. It will provide evidences for analyzing the flounder spermatogenesis process, as well as cilium development.

Abbreviations

dnali1, dynein axonemal light intermediate chain 1; ORF, open reading frame; 5'UTR, 5' untranslated region; 3'UTR, 3' untranslated region; TL, total length; MS222, tricaine methane sulfonate; HE, hematoxylin/eosin; RT-PCR, reverse transcription polymerase chain reaction; RACE, rapid amplification of cDNA ends; qPCR, real-time quantitative polymerase chain reaction; IDA, inner dynein arms ; ODA, outer dynein arms.

Declarations

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Compliance with ethical standards

Conflict of interest

Authors declare no conflict of interests.

Ethical approval

The study was conducted in accordance with the guidelines and regulations established and approved by the ethics committee of Institute of Oceanology, Chinese Academy of Sciences. All of applicable international, national, and institutional guidelines for the care and use of animals were followed by the authors.

Consent to participate

All the authors listed have approved the manuscript that is enclosed.

Consent for publication

The manuscript is approved by all authors for publication.

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Figures

Figure 1

The analyses of gene structure and phylogenetic tree a, Genomic structure of the flounder *dnali1*. Colorful boxes indicate exons, and introns are between them. Modules of the same color in the last three exons mean the same sequence. b, Alternative splice of 5' UTR. There are two different alternative splicing variants in 5'UTR. c, Multiple sequence alignment of the *dnali1*. The identical and similar amino acids are highlighted in solid black and red, respectively. DNAMAN software was used to carry out the multiple sequence alignment. The red box denotes the conserved domain. d, Phylogenetic tree was built using the neighbor-joining method. Values at the branch points indicate the percentage of 1000 bootstrap replicates supporting the division. The flounder is marked. Animal scientific names and GenBank accession Nos. are listed in Table S1.

Figure 2

Genomic sequences of dnali1 The red letters indicate the start codon and stop codon. The underlined nucleotides are the repeated sequences. Lowercase letters and “...” mean introns. The letters with box are specific primers for the genomic sequence confirmation.

Figure 3

See the manuscript file for the complete figure caption.

Figure 4

Expression of the flounder *dnali1* in different tissue and gonads a, Tissue distribution. M, marker; B, brain; St, stomach; H, heart; E, eye; Mu, muscle; Sp, spleen; HK, head kidney; I, intestine; K, kidney; G, testis/ovary; Gi, gill; L, liver. NC, negative control. b, Expression in the gonads. Different letters indicate significant differences at gonadal development stages of the testis and ovary ($P < 0.05$). * represents significant difference between the testis and ovary at same gonadal development stage ($P < 0.05$); ** represents extremely significant difference ($P < 0.01$).

Figure 5

3'UTR structure, schematic illustration of constructed plasmids, and GFP expression regulated by the flounder *dnali1* 3'UTR in zebrafish a, 3'UTR structure. 3'UTR sequences could be divided into two parts: fragments I and II. Fragment I represents the rest of the first repeat segment behind termination codon, fragment II contains the whole second 922 bp repeat segment, and fragment III represents whole 3'UTR (fragments I and II). b, Schematic illustration of the constructed plasmids. Different color boxes indicate

elements in the plasmids. c, GFP expression in the control, fragment Δ , fragment Δ , and fragment Δ groups. The red arrow indicates positive signal.

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