Gene 591 (2016) 183–190

Contents lists available at ScienceDirect

Gene

journal homepage: www.elsevier.com/locate/gene

Research paper

Molecular characterization and expression pattern of a germ cell marker gene *dnd* in gibel carp (*Carassius gibelio*)

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ARTICLE INFO

Article history:
Received 13 April 2016
Received in revised form 27 June 2016
Accepted 9 July 2016
Available online 11 July 2016

Keywords:
Dead end
Primordial germ cell
Germ cells
Oogenesis
Spermatogenesis

ABSTRACT

As a germ cell marker gene, *Dead end* (*dnd*) has been identified and characterized in many vertebrates. Recently, we created a complete germ cell-depleted gonad model by the *dnd*-specific morpholino-mediated knockdown approach, and revealed sex-biased gene expression alteration through utilizing unisexual gynogenetic superiority in polyploid gibel carp. However, *dnd* and its expression pattern are still unclear in the gibel carp. In this study, we further analyzed molecular characterization of gibel carp *dnd* and its dynamic expression pattern during gametogenesis and embryogenesis. Similar to other homologs in vertebrates, gibel carp *dnd* contains a conserved RRM motif and five other motifs, and is highly evolutionary conserved in genomic organization and neighborhood gene synteny. RT-PCR and Western blot analyses showed its gonad-specific expression intensively in testis and ovary. Section *in situ* hybridization (SISH) and immunofluorescence localization revealed its dynamic expression pattern specific to oogenic cells and spermatogenetic cells during oogenesis and spermatogenesis. Moreover, its temporal and spatial distribution specific to PGCs were also demonstrated by RT-PCR and whole mount *in situ* hybridization (WISH) during embryogenesis. Therefore, gibel carp *Dnd* is a conserved germ cell marker during gametogenesis, and its maternal transcript is also a useful marker for tracing PGC specification and migration.

1. Introduction

Dead end (*dnd*) is a germ cell marker gene in vertebrates, and encodes a RNA-binding protein that is a critical component for primordial germ cell (PGC) survival and migration (Weidinger et al., 2003). *Dnd* has been cloned and identified in many vertebrates, including mouse (Youngren et al., 2005), zebrafish (Weidinger et al., 2003), chicken (Aramaki et al., 2009), Xenopus (Horvay et al., 2006), medaka (Liu et al., 2009; Hong et al., 2016), loach (Fujimoto et al., 2010), goldfish (Goto et al., 2012), sterlet sturgeon (Linhartova et al., 2015), and Atlantic salmon (Wargelius et al., 2016). In most of examined teleost fishes, such as zebrafish, medaka, loach, goldfish, sterlet sturgeon, and Atlantic salmon, *dnd* is specifically expressed in PGCs, and the *dnd* translation block or depletion completely abolishes PGCs during early embryogenesis (Weidinger et al., 2003; Fujimoto et al., 2010; Goto et al., 2012; Hong et al., 2016; Wargelius et al., 2016). In some species, however, the *dnd* expression in adults appears to be different with sex. In Xenopus, *dnd* expression is restricted in ovary (Horvay et al., 2006), whereas in mouse, *dnd1*α is specifically expressed in testis (Bhattacharya et al., 2007). Similarly, a differential expression pattern of *dnd* between testis and ovary was also observed in turbot (Lin et al., 2013) and Pacific bluefin tuna (Yazawa et al., 2013). Therefore, the molecular characterization and expression pattern of *dnd* should be further revealed in various vertebrates.

Gibel carp, *Carassius gibelio*, is a unique polyploid cyprinid fish that is able to reproduce by unisexual gynogenesis (Xie et al., 2001; Dong et al., 2004; Yang and Gui, 2004). However, unlike most unisexual vertebrates (Avise, 2008), gibel carp has been revealed to have multiple reproduction modes including unisexual gynogenesis and sexual reproduction (Zhou et al., 2000; Gui and Zhou, 2010; Li et al., 2014a; Mei and Gui, 2015; Zhang et al., 2015), and a high male incidence has been also observed in many natural populations (Jiang et al., 2013; Li et al., 2016). Utilizing it as an evolutional development biology model for screening reproduction-related genes, many important genes, such as oocyte-specific and ovarian development-associated genes *C-type lection* (Dong et al., 2004), *C1q-like* (Mei et al., 2008, 2014) and *spindlin* (Sun et al., 2010), oocyte-specific H2A variant *h2af1o* (Wu et al., 2009; Yue et al., 2013) and stem-loop binding protein-encoded gene *slbp2* (Liu et al., 2015a), testis determination-related gene *Dmrt1* (Li et al.,

Abbreviations: bp, base pairs; *dnd*, *dead end*; hpf, hour post fertilization; DAPI, 4′,6-diamidino-2-phenylindole; PBS, phosphate buffer solution; PGCs, primordial germ cells; RRM, RNA recognition motif; RT-PCR, real time-polymerase chain reaction; SISH, section *in situ* hybridization; WISH, whole mount *in situ* hybridization.

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http://dx.doi.org/10.1016/j.gene.2016.07.027
0378-1119/

2014a), and germ cell marker genes *Vasa* and *Dazl* (Xu et al., 2005; Peng et al., 2009), have been identified and functionally characterized in gibel carp. In a previous study, we have utilized the superiority of unisexual gynogenesis in gibel carp to create a complete germ cell-depleted gonad model by a *dnd*-pecific morpholino-mediated knockdown approach, and revealed that the complete depletion of primordial germ cells in the gynogenetic all-female fish leads to sex-biased gene expression alteration and sterile all-male occurrence (Liu et al., 2015a). However, molecular characterization of the *dnd* has been not reported and its expression pattern is still unclear in gibel carp. In this study, we further analyzed molecular characterization of gibel carp *dnd* and its dynamic expression pattern during gametogenesis and embryogenesis.

2. Materials and methods

2.1. Experimental fish collection

Gibel carp was collected from Guanqiao experimental station of the Institute of Hydrobiology, Chinese Academy of Sciences. Inducing spawning and embryos culture was performed as described previously (Liu et al., 2015a).

2.2. Full length cDNA cloning and sequence analysis

According to the highly conserved regions of teleost *dnd* homologs, a pair of primers (*CgDnd* F1 and *CgDnd* R1) was designed as shown in Table 1. An expected 211 bp fragment was obtained from gibel carp ovary SMART cDNA library. And the full of length of *CgDnd* cDNA was further obtained by 3′ and 5′ RACE, using *CgDnd* F2, *CgDnd* R2 and the adaptor primer (5′-AAGCAGTGGTAACAACGCAGAGTAC-3′). The RACE-PCR was performed as preciously described (Liu et al., 2015b). The cDNA sequence has been submitted to GenBank with accession no. KP641680. Multiple alignments of DND proteins were performed with BioEdit software. The unrooted phylogenetic tree was constructed with Mega 6.0 by bootstrap analysis using neighbor-joining (1000 replicates).

2.3. Identification and sequence analysis of BAC clone with CgDnd

To isolate the BAC clone with *CgDnd* from gibel carp genome BAC library, two levels of BAC DNA pools were constructed for BAC clone isolation based on PCR strategy as described previously (Li et al., 2014b), and the primers were shown in Table 1. *CgDnd* BAC clone was completely sequenced and analyzed at Beijing Genomics Institute as described previously (Peng et al., 2009). The 7251 bp *CgDnd* genomic sequence has been submitted to GenBank with accession no. KX022948. Genscan software was used for gene prediction on the BAC sequence.

Table 1
Primers used in this study.

Primers	Sequences(5′-3′, T7 promoter are shown in red)	Application
CgDnd F1	GAACTTCAGTGGGCAGAACC	RT-PCR
CgDnd R1	GTCAGAGATCATTCGCAGCA	
CgDnd F2	CCTACGCTAAGTATGGTGACC	RACE
CgDnd R2	GCACCGTCAGCAACTCCCCCTGAT	
CgDnd G1F	CAGGTGAGCGGGTTTATTTAC	BAC clone isolation
CgDnd G1R	GGACCACCATATTTCCTCTGC	
CgDnd G2F	AGTTGTCATGTTTTCATCAGTT	
CgDnd G2R	TTGCCAGCCTGGAGTTAAAGA	
β-actin-F	CGAGCTGTCTTCCCATCCA	
β-actin-R	CAACGTAGCTGTCTTTCTG	
CgDnd F3	AGGAGTGAAATTTGAGCGCG	Anti-sense probe synthesis
CgDnd R3	GGATCCTAATACGACTCACTAT AGTCAGAGATCATTCGCAGCA	
CgDnd F4	GGATCCTAATACGACTCACTATAA GGAGTGAAATTTGAGCGCG	Sense probe synthesis
CgDnd R4	GTCAGAGATCATTCGCAGCA	

2.4. RNA isolation and RT-PCR

Using SV (Spin or Vacuum) total RNA isolation system (Promega), the total RNA was extracted from adult tissues or 30 embryos of different developmental stages. The cDNA library was constructed as previously described (Li et al., 2014a). The real-time quantitative PCR (RT-qPCR) was performed as previously (Liu et al., 2015a). The PCR cycling conditions were: 94 °C (2 min) for heat denaturing, followed by 40 cycles of 94 °C (15 s), 57 °C (15 s), 72 °C (20 s), and additional 72 °C (2 min). *β-actin* was used as an internal control. All the samples were analyzed in triplicates, and relative expression level of target gene was calculated with the $2^{-\Delta CT}$ methods (Liu et al., 2015a; Yue et al., 2013).

2.5. Polyclonal antibody preparation and Western blot analysis

Polyclonal antibody specific to the *Cg*Dnd was produced against the 14 amino acids peptide (GGPAGLRDETMPRA) that locates at 270–283 amino acid site in the C-terminus CR3 motif. The peptide was conjugated to the KLH (Keyhole Limpet Hemocyanin) peptide to immunize the rabbit to get the polyclonal antibody (Genscript company). Western blot analysis was performed as previously described (Dong et al., 2004).

2.6. Immunofluorescence staining

Immunofluorescence localization was performed as described previously (Sun et al., 2010; Liu et al., 2015a). In brief, samples were sectioned at 8 μm for ovary and 5 μm for testis. The cryostat sections were dried at 37 °C for 1 h, and rehydrated in PBS once and fix in 4% paraformaldehyde (PFA) 15 min at room temperature, and washed by PBS 5 min three times, then blocked for 1 h with 5% milk preventing non-specific binding. The sections were then incubated with anti-*Cg*Dnd antibody (1:200) at 4 °C overnight. After washing with 0.1% Tween 20 in PBS 10 min six times, the sections were incubated 1 h with secondary antibody contains 1:200 diluted FITC-conjugated goat anti-rabbit IgG (H + L) and DAPI (1 μg/ml). The sections were washed 10 min six times, and finally observed with a Leica confocal laser scanning microscope (Leica SP8).

2.7. Section in situ hybridization (SISH)

Cryostat section *in situ* hybridization was performed as previously described (Xia et al., 2007). Briefly, ovary and testis were dissected and fixed in 4% PFA/PBS, pH 7.4 at 4 °C overnight. Samples were washed in PBS at room temperature three times and each time for 10 min, immersed in 30% sucrose, embedded in optimal cutting temperature (O.C.T.), sectioned at 8 μm for ovary and 5 μm for testis, and dried at 37 °C for 1 h. Sections were rehydrated in PBS for 5 min three times, then treated with 10 μg/mL proteinase K for 5 min, and refixed in 4% PFA for 30 min. After that, the slides were hybridized at 60 °C for 16 h. After hybridization, the slides were washed at 65 °C in 50% of formamide/5XSSCT 30 min twice, 0.2XSSCT 30 min twice, and MABT 30 min twice at room temperature. The sections were blocked with 2% blocking reagent in MABT for 3 h at room temperature, and incubated in alkaline phosphatase conjugated anti-DIG antibody (1:5000 diluted in blocking solution) at 4 °C overnight. Finally, the slides were washed in MABT for 15 min five times, and the signal was detected using NBT/BCIP as the chromogenic substrate.

2.8. Whole mount in situ hybridization (WISH)

A 650 bp digoxigenin-labeled antisense *CgDnd* probe containing RRM domain probe was produced as previously described (Xiao et al.,