Comparative Biochemistry and Physiology, Part B 177–178 (2014) 56–64

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Molecular characterization and functional divergence of two *Gadd45g* homologs in sex determination in half-smooth tongue sole (*Cynoglossus semilaevis*)

Wan-Jun Liu [a,b], Li-Yan Zhang [a], Chang-Wei Shao [a], Na Wang [a], Kun Liu [a], Hai-Shen Wen [b], Ning Zhang [a], Zhong-Dian Dong [b], Jun-Jie Zhang [a], Song-Lin Chen [a,*]

[a] Yellow Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences, Qingdao 266071, China
[b] Fisheries College, Ocean University of China, 5 Yushan Road, Qingdao 266003, China

ARTICLE INFO

Article history:
Received 26 January 2014
Received in revised form 1 May 2014
Accepted 1 September 2014
Available online 8 September 2014

Keywords:
Cynoglossus semilaevis
Gadd45g
Sex differentiation

ABSTRACT

The growth arrest and DNA-damage-inducible protein 45 gamma (Gadd45g) is known to play a major role in embryonic development and sex determination. In this study, two *Gadd45g* genes were isolated from half-smooth tongue sole (*Cynoglossus semilaevis*). Using chromosomal fluorescence in situ hybridization (FISH), *Gadd45g1* and *Gadd45g*2 were located on the W and Z chromosomes, respectively. The full-length cDNA sequences of *Gadd45g1* (1270 bp) and *Gadd45g2* (1181 bp) were predicted to contain a 480-bp coding sequence that could encode a protein of 159 amino acids residues. A phylogenetic tree showed that the predicted Gadd45g1 and Gadd45g2 amino acid sequences clustered closely in one branch. It is proposed that *Gadd45g1* and *Gadd45g2* are paralogous genes derived from the divergence of the sex chromosome. Ka/Ks ratios indicated that *Gadd45g1* and *Gadd45g2* may have undergone a high number of mutations and have a divergence time of only about 68,000 years, although *Gadd45g* homologs are highly conserved. The qRT-PCR demonstrated that *Gadd45g1* and *Gadd45g2* were highly expressed in ovary, and negligibly expressed in testis of male and neo-male. During development of the ovary (from 80 to 150 days), the expression levels of both genes reached high levels. *Gadd45g1* was also highly expressed at 50 days, the stage just before gonad differentiation in *C. semilaevis*. All these findings imply functional divergence of the two *Gadd45g* homologs; *Gadd45g1* may be necessary for sex differentiation in the early stage of gonad development, and then *Gadd45g1* and *Gadd45g2* maintain ovary development and the female character of half-smooth tongue sole.

1. Introduction

Growth arrest and DNA-damage-inducible protein 45 (*Gadd45*) genes encode a small family of multifunctional stress response proteins (Fornace et al., 1988) that mediate diverse cellular processes, including DNA repair, apoptosis, cell cycle arrest, senescence (Hollander and Fornace, 2002; Liebermann et al., 2011), and DNA demethylation (Sheikh et al., 2000; Zhan, 2005; Barreto et al., 2007; Niehrs and Schäfer, 2012). Gadd45 family members Gadd45a, Gadd45b, and Gadd45g appear to have overlapping but non-identical functions and binding partners, and are induced by different stimuli (Liebermann and Hoffman, 2008).

Recently, many studies have focused on the important role of Gadd45g in embryonic development and sex determination. *Gadd45g* was found to be expressed in primary neuron precursors and was reported to promote differentiation by regulation of cell cycle exit in medaka fish (*Oryzias latipes*) and the African clawed frog (*Xenopus laevis*) (de la Calle-Mustienes et al., 2002; Candal et al., 2004). *Gadd45g* genes were also found to be involved in vertebrate neurogenesis (Kaufmann et al., 2011). However, previous studies in mice (*Mus musculus*) found that *Gadd45g* genes were dispensable for embryonic development, because adult mutant mice were viable and did not present obvious malformations (Hollander et al., 1999; Hoffmeyer et al., 2001; Gupta et al., 2006). In-depth studies of *Gadd45g*-mutant mice (Hoffmeyer et al., 2001; Cai et al., 2006; Bouma et al., 2007; Bogani et al., 2009; Warr and Greenfield, 2012) revealed a specific role for *Gadd45g* in mammalian sex determination. It was determined that Gadd45g, but not Gadd45a or Gadd45b, was necessary for activation of the male sex-determining pathway in mice (Johnen et al., 2012) and *Gadd45g*-mutant mice showed complete male-to-female sex reversal. A signaling cascade required for sex-determining region Y (*Sry*) transactivation was also found (Gierl et al., 2012; Warr et al., 2012).

The half-smooth tongue sole (*Cynoglossus semilaevis*) is an increasingly important marine flatfish of potentially great aquaculture value

* Corresponding author at: Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Science, Nanjing Road 106, 266071 Qingdao, China. Tel.: +86 532 85844606; fax: +86 532 85811514.
E-mail address: chensl@ysfri.ac.cn (S.-L. Chen).

http://dx.doi.org/10.1016/j.cbpb.2014.09.001
1096-4959/

in China (Liu et al., 2005). It exhibits prominent sexually dimorphic growth (Ji et al., 2011) in which females grow two to three times faster than males. As a result, female half-smooth tongue sole are favored for cultivation; however, female-to-male sex reversal in this species (Ji et al., 2010) can reduce the proportion of phenotypic females, causing huge financial losses. As a result, the physiological mechanism of sex reversal in *C. semilaevis* is of great interest and still needs to be clarified.

Here, we cloned two *Gadd45g* homologous genes (*Gadd45g1* and *Gadd45g2*) in *C. semilaevis*, and analyzed the physical location, sequence characteristics, evolution, and expression patterns of these two genes. The aim of this study was to reveal the physiological functions and relationship between *Gadd45g1* and *Gadd45g2* to determine their effect on sex determination in *C. semilaevis*.

2. Materials and Methods

2.1. Fish and sampling

The half-smooth tongue soles (*C. semilaevis*) used in the experiments were obtained from Huanghai Aquaculture Ltd. (Haiyang, Shandong Province, China). One-year-old sexually mature fish (three individuals of each gender) were randomly sampled. Spleen, skin, heart, liver, gill, kidney, whole brain, intestine, ovary or testis, muscle, and blood tissues (11 tissues in all), were collected and snap-frozen in liquid nitrogen, and then stored at −80 °C. Additionally, gonads at different developmental stages were collected from larvae from the same family as the sexually mature fishes and stored at −80 °C. The gonads were collected from three individuals of each gender at 20, 35, 50, 65, 80, 95, 120, and 150 days after hatching. To determine the genetic sex of the fish in our study, their fins were collected and stored in 100% ethanol for DNA extraction.

2.2. RNA extraction and genetic sex identification

Total RNA was extracted from the frozen tissues using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA synthesis was conducted using a PrimeScript™ RT regent Kit with gDNA Eraser (TAKARA, Dalian, China).

Genomic DNA was extracted from fins using a phenol-chloroform protocol (Chen et al., 2007). Female-specific PCR primers, CseF382F and CseF382R (Hu and Chen, 2013), were used to amplify the DNA for genetic sex identification. The PCR products were separated on 1% agarose gels and samples with a 291-bp band were identified as genetic females, while samples with no band were identified as genetic males. The gonad histology was carried out as described (Chen et al., 2008).

Neo-males were identified as individuals that were genetic females, but phenotypic males.

2.3. Primer design and fluorescence in situ hybridization (FISH)

To validate the genome sequences in the extracted RNA and to determine the physical location of *Gadd45g1* and *Gadd45g2* in the *C. semilaevis* genome, two pairs of updated primers (Table 1) were designed based on the *Gadd45g* sequence from the whole-genome sequence results (Chen et al., 2014). All the primers used in our study (Table 1) were designed by Primer Premier 5.0 software (Premier Biosoft, Palo Alto, CA, USA).

The positive clone corresponding to the target gene was screened using updated primers from the *C. semilaevis* BAC library (Shao et al., 2010) to enlarge cultivation. Plasmids were extracted from the positive clone using a Mini Plasmid Kit (Biomed, Beijing, China), and then used to prepare FISH probes using DIG-Nick Translation Mix (Roche, Basel, Switzerland). Preparation of the chromosomes of the mature male and female *C. semilaevis* was finished as described previously (Xie et al., 2012).

Table 1
Primers used in this study.

Group	Primers name	Sequences (5′ to 3′)
Sex identification primers	CseF382F	ATTCACTGACCCCTGAGAGC
	CseF382R	TGGCACCATCATTGTAAAACTA
Updated primers	Gadd45g1-A	GCTTTGGGAGAGTGATGCTG
	Gadd45g1-S	CGCCAAGAAGCAGGACTACC
	Gadd45g2-A	CTGGAAGGGTGATGCTGGGC
	Gadd45g2-S	CACGGACAAGACAACACGATGGA
RACE primers	Gadd45g1-3′R	GCGAGGAGAGCCGCAGCGTGTA
	Gadd45g1-3′N	GTGCCCAGCATCACTCTCCCAA
	Gadd45g1-5′R	GCAGTGCGGGTCCTTGGGCTCG
	Gadd45g1-5′N	GCACCCTGTCACTGTTTTCCGTC
	Gadd45g2-3′R	CGCCAAGAATCGGGACTACCTGACG
	Gadd45g2-3′N	AATGTTGACCCAGACAGCGTG
	Gadd45g2-5′R	TCCAACCGTCAGGTAGTCCCGATTC
	Gadd45g2-5′N	TTTTCCATCGTGTTGTCTTGTCC
	UPM-long	CTAATACGACTCACTATAGGGCAAGCAG TGGTATCAACGCAGAGT
	UPM-short	CTAATACGACTCACTATAGGG
	NUP	AAGCAGTGGTATCAACGCAGAGT
qRT-PCR primers	Gadd45g1-qrt-a	ACACTGTTGCAGGGGAGGAT
	Gadd45g1-qrt-s	TGGTGAAAAGAAAGTCAAGGAGC
	Gadd45g2-qrt-a	GGGTGAAGTGGATCTGGAGC
	Gadd45g2-qrt-s	AAGAATCGGGACTACCTGACG
	Actin-a	GAGTAGCCACGCTCTGTC
	Actin-s	GCTGTGCTGTCCCTGTA

The FISH probes were applied to the chromosome slide and hybridized with target DNA, then incubated with Goat Anti-DIG antibody (Roche) and stained stepwise with secondary fluorescein isothiocyanate (FITC)-labeled donkey anti-sheep IgG antibodies (Roche). After air-drying in the dark, the slides were counterstained with fluorescence quenching agent with propidium iodide (PI) (Sigma-Aldrich, Santa Clara, CA, USA) and covered with a cover slip. The slides were examined under a fluorescence microscope (Nikon Eclipse 80i) equipped with an ×60 oil immersion lens and photographed using a Nikon camera (Nikon Digital Sight Ds-Fi2).

2.4. RACE

Two pairs of RACE-specific primers were designed for 5′- and 3′-RACE, based on the genomic DNA sequences that were identified from the whole-genome sequencing results (Chen et al., 2014). To obtain the full-length sequences of *Gadd45g1* and *Gadd45g2* genes, 5′- and 3′-RACE were performed using a SMARTer™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA). Here, to illustrate the method, the full-length cloning of one of the genes (*Gadd45g1*) is described in detail. Gadd45g1-5′R/3′R primers and universal primer 10*UPM were used for touchdown PCR under the following conditions: denaturation at 94 °C for 5 min, followed by 14 cycles (94 °C for 30 s, first cycle 72 °C for 1 min and subsequently the temperature was reduced by −0.5 °C per cycle, and 72 °C for 1 min), 30 cycles (94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min), and then at 72 °C for 10 min. The same method was used for *Gadd45g2*. The 5′- and 3′-RACE product were diluted 100 times with RNase-free ddH_2O and used as template for a nested PCR reaction using Gadd45g1-5′N/3′N primers and nested universal primer NUP under the following conditions: 94 °C for 5 min, followed by 30 cycles (94 °C for 30 s, 60 °C for 30 s, and 72 °C for 1 min), and 72 °C for 10 min.PCR reactions were performed using a Veriti™ 96-well Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR products were separated on 1.5% agarose gel, purified with Zymo Gel DNA Recovery Kit (Zymo Research, Irvine, CA, USA), ligated into a pMD-18 T vector (TaKaRa) and propagated in the *E. coli* Top10 (Tiangen, Beijing, China). Three positive clones were selected for sequencing. The same method was used for the other gene, *Gadd45g2*.