Comparative Biochemistry and Physiology, Part B 150 (2008) 278–283

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part B

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

Identification of estrogen receptor α gene polymorphisms by SSCP and its effect on reproductive traits in Japanese flounder (*Paralichthys olivaceus*)

Feng He, Hai Shen Wen *, Shuang Lin Dong, Lian Shun Wang, Cai Fang Chen, Bao Shi, Xing Jiang Mu, Jun Yao, Yu Guo Zhou

Key Laboratory of Mariculture Ministry of Education, Ocean University of China, Qingdao 266003, People's Republic of China

ARTICLE INFO

Article history:
Received 19 December 2007
Received in revised form 20 March 2008
Accepted 20 March 2008
Available online 28 March 2008

Keywords:
Japanese flounder
Estrogen receptor α
Diplotype
SNPs
Reproductive traits

ABSTRACT

Estrogen receptor (*ERα*) modifies the expression of genes involved in cell growth, proliferation and differentiation through binding to estrogen response elements (EREs) located in a number of gene promoters, so the *ERα* gene is considered as an important factor affecting reproductive endocrinology in Japanese flounder (*Paralichthys olivaceus*). In this study, twelve single nucleotide polymorphisms (SNPs) within eight CDS exons and 1 kb of 3′-UTR of the *ERα* gene were tested to association with four reproductive traits in a population of 119 Japanese flounder individuals with polymerase chain reaction-single stranded conformational polymorphism (PCR-SSCP). The association analysis of SNPs within Japanese flounder *ERα* gene with the reproductive traits was carried out using General Linear Model (GLM) estimation. Results indicated that two SNPs in the exon4 of *ERα* gene, P1 (A803G and C864T), were significantly associated with hepatosomatic index (HSI) ($P<0.05$) in female Japanese flounder. Other ten SNPs in 3′-UTR associated to serum 17β-estradiol (E_2) and HSI showed that P2 (A1982T) was significantly associated with E_2 ($P<0.01$) and P3 (A2149G, 2181TTACAAG2182 insertion or deletion, T2324G, A2359G and G2391A) was significantly associated with HSI ($P<0.05$) in female Japanese flounder. However, P2 (A1982T) and P4 (G2256T, T2294C, T2309G and A2333T) had significant effects on E_2 ($P<0.05$ and $P<0.01$, respectively) in male Japanese flounder. In addition, there were significant associations between diplotype D1 based on fourteen SNPs and reproductive traits. The genetic effects for HSI (female) or E_2 (male) of diplotype D1 were significantly higher than those of other eight diplotypes ($P<0.05$), respectively. Our findings implied that P1 of *ERα* gene affecting the reproductive traits could be a potential QTN (quantitative trait nucleotide) which would be useful genetic marker in the selection of some reproductive traits for its in Japanese flounder.

1. Introduction

ER is expressed in reproductive as well as nonreproductive tissues (Gustafsson, 1999; Muramatsu and Inoue, 2000; Albertazzi and Purdie, 2001) and its role is implicated in the control of proliferation, differentiation, and development of many tissues (Henderson and Feigelson, 2000; Saji et al., 2000; Albertazzi and Purdie, 2001). A major role of the ER signaling pathway in females is the development of normal functions of the reproductive tract such as the growth of uterus, secondary sex characteristics and reproductive behavior (Muramatsu and Inoue, 2000). Likewise in males, the ER pathway is necessary in the normal development and physiology of reproductive organs (Eddy et al., 1996).

Single nucleotide polymorphisms (SNP), one base change including deletion, insertion, and substitution, would greatly influence gene expression and the functions of proteins. Polymorphisms and mutations in various genes have been shown to cause changes in the function of their resultant mutated proteins, such as the inactivation of DGAT1 (Grisart et al., 2002; Winter et al., 2002; Thaller et al., 2003). On the contrary, polymorphisms can also lead to enhanced protein activation such as CYP1B1 (Shimada et al., 1999; Hanna et al., 2000) and CYP1A1 (Zhang et al., 1996). With regard to polymorphism of *ERα* gene, polymorphisms of various regions of *ERa* have been found to be associated with various characteristics and diseases in humans. Intron 1-PvuII polymorphism was found to be associated with lower bone mineral density (Kobayashi et al., 1996; Ongphiphadhanakul et al., 1998; Willing et al., 1998; Kurabayashi et al., 1999), shorter height (Lorentzon et al., 1999), and risk of fall among postmenopausal women undergoing hormone replacement therapy (Salmen et al., 2002; Sowers et al., 2006). Those association studies show that observed phenotypic associations may be explained by linkage disequilibrium with *ERα* polymorphisms. Although significant numbers of SNPs in the aquaculture species have been few reported by He et al. (2003), Sarah and Gloria (2007) and Hayes et al. (2007), there were no association studies between mutations and traits. However, there are no reports

* Corresponding author. Tel./fax: +86 532 82031825.
E-mail address: wenhaishen@ouc.edu.cn (H.S. Wen).

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doi:10.1016/j.cbpb.2008.03.013

describing polymorphisms for the Japanese flounder *ERα* gene, and association studies between diversities and reproductive performances are also scarce.

Japanese flounder (*Paralichthys olivaceus*) is a teleost fish which has XX (female)/XY (male) sex determination system (Tabata, 1991). The genetic females can be completely sex reversed to phenotypic males when the larvae are reared at a high water temperature (27 °C) during the sex differentiation period (Kitano et al., 1999). Therefore, the flounder provides an excellent model to study molecular mechanism of functional candidate gene mutation affecting reproductive straits under the same temperature (< 27 °C) condition. In Japanese flounder, *ERα* has been cloned (GenBank Accession No. AB070629). Japanese flounder *ERα* cDNA spans 2796 bp and contains 8 exons and approximately 1 kb 3′ non-coding region, which encodes a mature protein with 578 amino acids.

It is generally accepted that the steroid hormones, testosterone (T) and E_2, play an important role in fish gonad development (Struessmann and Nakamura, 2002). Functions of T and E_2 are transited by ER and AR subtypes into regulation of reproductive and metabolic homeostasis (Goksoyr, 2006; Tabb and Blumberg, 2006). The gonadosomatic index (GSI) is a gross quantitative indicator of gonad condition and represents the simplest way to measure changes in size and weight of this organ in relation to total weight of the organism (Hervey et al., 2006). In this study, we have selected four measured reproductive traits, namely testosterone (T), 17β-estradiol (E_2), hepatosomatic index (HSI) and gonadosomatic index (GSI), tested polymorphisms in the Japanese flounder *ERα* gene and determined if or not mutation detected in Japanese flounder *ERα* gene could be relative to reproductive traits.

Single-stranded conformational polymorphism (SSCP) analysis is one of the simplest, most reliable, and most sensitive methods for detection of mutations based on PCR (Orita et al., 1989; Sheffield et al., 1993). We have optimized the SSCP procedure to detect single nucleotide polymorphisms (SNPs) and used this method to evaluate polymorphisms of *ERα* and their association with reproductive traits.

2. Materials and methods

2.1. Animals

Japanese flounder (*Paralichthys olivaceus*), 65 female (239.23 ± 74.93 g) and 54 male (230.4 ± 70.74 g), were reared at natural sea water temperature (20 ± 0.5 °C) in our laboratory under the same rearing and management conditions. Fish were decapitated and the gonads and livers were removed and weighed when fish were reared at six months. Four reproductive traits, namely, testosterone (T), 17β-estradiol (E_2), hepatosomatic index (HSI) and gonadosomatic index (GSI), were used for association analysis. Table 1 presented the mean and standard deviations of four traits.

Table 1
Means and standard deviations of reproductive traits[1]

Traits	Mean	SD[1]
T[2] (pg/mL) (female)	17.64	7.36
E_2^3 (pg/mL) (female)	6.31	3.74
HSI[4] (female)	1.55	0.567
GSI[5] (female)	0.155	0.122
T[2] (pg/mL) (male)	17.05	14.58
E_2^3 (pg/mL) (male)	6.67	5.24
HSI[4] (male)	2.51	1.68
GSI[5] (male)	0.454	0.107

1 = Standard deviation.
T[2] = Testosterone.
E_2^3 = 17β-estradiol.
HSI[4] = Hepatosomatic index.
GSI[5] = Gonadosomatic index.

Table 2
Primer sequences and information of Japanese flounder *ERα* gene

Names	Sequences	Length (bp)	Tm (°C)	Target region
Primer1	5-AGCACTCTCAGACCTGGAGA-3 5-GCTGACTGGACCTGTACACA-3	359	57	Exon1
Primer2	5-CCAGTAACCAGAGAGGACCA-3 5-CTGGATGCTCCTCTTGAAGA -3	201	58	Exon2
Primer3	5-TCACAATGACTACATGTGCC-3 5-CTCCTTTCATCATGCCAACT-3	116	56	Exon3
Primer4	5-GACCGCAGCCATGTCTTA-3 5-GTGAGCAGGGTCATCATG-3	266	58	Exon4
Primer5	5-TTCATGACCAGGTTCAGCT-3 5-TGTCCAGTATGAGGTCCTG-3	120	56	Exon5
Primer6	5-ATGGCTGAGATCTTCGAC-3 5-CCGAGTTGAGAAGGATGA-3	109	55	Exon6
Primer7	5-GCACAACACCGCAGCCGT-3 5-GGAGAGCAGCAGGAGCAG-3	130	62	Exon7
Primer8	5-AGGCATGGAGCACCTCTA-3 5-GTGTCCTGAACTGGCTGA-3	220	57	Exon8
Primer9	5-ACACCAGAGAATGAAAGAGG-3 5-GTCCCTTAAACTCCAGATGA -3	308	57	3′UTR
Primer10	5-TCATCTGGAGTTTAAGGGAC-3 5-TGGATGTGAAGTTACCCAAC-3	309	57	3′UTR
Primer11	5-GTTGGGTAACTTCACATCCA-3 5-AAGAAATGACAGTCGGGTCA-3	310	57	3′UTR

2.2. Hepatosomatic index (HSI) and gonadosomatic index (GSI)

The hepatosomatic index or gonadosomatic index (%) of each animal was calculated as the ratio of the gonad or liver wet weight to the whole body net weight. Gonadosomatic or hepatosomatic index = (gonad or liver weight / (body weight − viscera weight)) × 100 according to McRae and Mitchell (1995) and Sagi et al. (1996).

2.3. T and E_2 radioimmunoassay assays

The blood was sampled by puncturing the caudal vasculature with a 25-gauge 1.3-cm needle attached to a 1.0-mL disposable syringe. Blood samples were allowed to clot on ice for several hours; serum was then separated by centrifugation (16,000 *g*) for 5 to 7 min and stored at − 40 °C. Testosterone and estradiol-17β were quantified by ^{125}I radioimmunoassay, using kits from Diagnostic Products Corporation (Tianjin Nine Tripods Medical & Bioengineering Co., Ltd., Sino–US joint-venture enterprise). Steroids were assayed directly on the serum and antisera are highly specific with an extremely low crossreactivity to other naturally occurring steroids, the crossreactivity was less than 0.1% to most circulating steroids. Intraassay variability was 7.4% for the estradiol-17β assay and 8.0% for the testosterone assay. Any samples with coefficient of variation higher than 10% were not included in the analyses. The assay sensitivity reached to 20 pg/mL for T and 4 pg/mL for E_2 by kit protocol (Wen et al., 2006).

2.4. PCR-SSCP analysis

Genomic DNA was isolated from blood samples by the phenol-chloroform method. Eleven pairs of primers were designed to amplify eight exons and about 1 kb 3′-UTR of Japanese flounder *ERα* according to its cDNA sequence (GenBank Accession No. AB070629) using the Oligo6.0 software (Table 2). PCR reactions were carried out in a total of 25 μL volume including 50 ng of genomic DNA, 0.20 mM each dNTP, 2.5 mM $MgCl_2$, 0.20 mM primers and 0.5 U Taq DNA polymerase. Amplification condition was 94 °C for 5 min followed by 35 cycles of 94 °C for 45 s, 57 °C for 45 s, and 72 °C for 45 s and a final extension at 72 °C for 10 min. The PCR products of *ERα* were genotyped by single-stranded conformation polymorphism (SSCP). PCR products (2 μL) of each individual were mixed with 5 μL denaturing buffer (98% formamide, 0.09% xylene cyanole FF, and 0.09% bromophenol blue), and then denatured at 94 °C for 5 min, followed by a rapid chill on ice for