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Molecular characterization, tissue distribution, and mRNA expression profiles of two *Kiss* genes in the adult male and female chub mackerel (*Scomber japonicus*) during different gonadal stages

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ABSTRACT

Kisspeptins, encoded by the *Kiss1* gene, have emerged as key modulators of reproduction in mammals. In contrast to the placental mammals, some teleosts express two *Kiss* genes, *Kiss1* and *Kiss2*. In the present study, full-length cDNAs of *Kiss1* and *Kiss2* in the chub mackerel were cloned and sequenced. Chub mackerel *Kiss1* and *Kiss2* cDNAs encode 105 and 123 amino acids, respectively. A comparison of the deduced amino acid sequences of chub mackerel *Kiss1* and *Kiss2* with those of other vertebrate species showed a high degree of conservation only in the kisspeptin-10 region (Kp-10). The Kp-10 of chub mackerel *Kiss1* (YNFNSFGLRY) and *Kiss2* (FNFNPFGLRF) showed variations at three amino acids. Tissue distribution analysis using quantitative real-time PCR (qRT-PCR) revealed that the *Kiss1* and *Kiss2* transcripts were expressed in different tissues of adult chub mackerel. In addition, their levels in the adipose tissue exhibited sexually dimorphic expression. Further, to have a basic understanding on the involvement of *Kiss1* and *Kiss2* in the seasonal gonadal development, their relative mRNA expression profiles in the brain, pituitary, and gonads at different gonadal stages were analyzed using qRT-PCR. *Kiss1* and *Kiss2* levels in the brain showed a differential expression profile between male and female fish. In males, *Kiss1* and *Kiss2* levels gradually decreased from the immature stage to spermiation and reached a minimal level during the post-spawning period. In contrast, *Kiss1* levels in the brain of females did not vary significantly among the different gonadal stages. However, *Kiss2* levels fluctuated as that of males, gradually declining from the immature stage to the post-spawning period. The pituitary *Kiss1* levels did not show significant fluctuations. However, *Kiss1* levels in the gonads were highly elevated during spermiation and late vitellogenesis compared to the immature and post-spawning period. These results suggest the possible involvement of two *Kiss* genes in the brain and *Kiss1* in the gonads of chub mackerel during seasonal gonadal development.

1. Introduction

Kisspeptins are peptide products of the *Kiss1* gene, belonging to the RFamide family, which share a common Arg-Phe-amide motif at their C-termini and are ligands for the G protein-coupled receptor 54 (GPR54), now named Kiss1r (Kotani et al., 2001; Muir et al., 2001; Roseweir and Millar, 2009). *Kiss1* was originally identified as a metastasis suppressor in malignant melanoma cells (Lee et al., 1996; Lee and Welch, 1997; Ohtaki et al., 2001). However, a major breakthrough was achieved in 2003, when multiple groups reported that humans and mice with either spontaneous or genetically targeted mutations in the *GPR54* gene exhibited impairments of reproductive function (Funes et al., 2003; de Roux et al., 2003; Seminara et al., 2003). Later, similar impairments were reported for *Kiss1*-knockout mice (Lapatto et al., 2007; d'Anglemont de Tassigny et al., 2007). These studies suggest that the kisspeptin/Kiss1r system plays a critical role in mammalian reproduction. In mammals, the Kiss1 precursor protein is cleaved into several mature peptides, including kisspeptin-54, -14, -13 and -10. All of these peptides share a distinct structural Arg-Phe-amide motif in their C-terminal regions (Bilban et al., 2004; Kotani et al., 2001; Ohtaki et al., 2001), and are reported to activate GPR54 with equal biopotency (Kotani et al., 2001; Muir et al., 2001; Ohtaki et al., 2001; Gottsch et al., 2004). Kisspeptins primarily act at the level of hypothalamic gonadotropin-releasing hormone (GnRH)

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neurons, which express *Kiss1r* (Murphy, 2005; Roa and Tena-Sempere, 2007). GnRH neurons are the final pathway through which the brain regulates the secretion of pituitary follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which initiate gonad development and maturation (Sherwood and Wu, 2005; Kauffman et al., 2007). The involvement of kisspeptin in this pathway has been demonstrated in different mammals through exogenous kisspeptin treatment, which elicits rapid increases in plasma levels of FSH and LH (Gottsch et al., 2004; Irwig et al., 2004; Dhillo et al., 2005; Navarro et al., 2005). These fundamental studies, followed by the publication of more than 150 research articles by different groups worldwide in recent years, further support the critical role of kisspeptins in key aspects of mammalian reproduction, such as the onset of puberty, seasonal reproduction, steroid feedback regulation, and metabolic control of fertility (Smith, 2008; Tena-Sempere, 2010a,b). However, kisspeptin research in non-mammalian vertebrates, particularly teleosts, is still scarce.

Recent studies in non-mammalian vertebrates have revealed the presence of *Kiss1* and *Kiss2* in an agnathan species, the sea lamprey (*Petromyzon marinus*) (Felip et al., 2009), some teleosts and amphibians (*Silurana tropicalis, Xenopus laevis)* (Felip et al., 2009; Li et al., 2009). In contrast, placental mammals express only *Kiss1* (Akazome et al., 2010). Moreover, the genomic sequence of a non-placental mammal, such as the platypus (*Ornithorhynchus anatinus*), reveals the presence of *Kiss1* and *Kiss2* genes (Felip et al., 2009). The freshwater teleosts zebrafish (*Danio rerio*; Biran et al., 2008; van Aerle et al., 2008; Kitahashi et al., 2009), medaka (*Oryzias latipes*; Kanda et al., 2008; Kitahashi et al., 2009), and goldfish (*Carassius auratus*; Li et al., 2009; Yang et al., 2010), and the marine teleost, European seabass (*Dicentrarchus labrax;* Felip et al., 2009), express *Kiss1* and *Kiss2* genes. In contrast, the genomes of fugu (*Fugu rubripes*), the green spotted puffer fish (*Tetraodon nigroviridis*), the stickleback (*Gasterosteus aculeatus*) and a reptile species, the green anole lizard (*Anolis carolinensis*) contain only *Kiss2* gene (Felip et al., 2009). In addition, the bird *Gallus gallus* lacks both *Kiss1* and *Kiss2* genes in the genome database (Felip et al., 2009). This diversification of *Kiss* genes has delayed their identification and characterization in non-mammalian vertebrates (Zohar et al., 2010). The recent nomenclature recommended for *Kiss1* and *Kiss1r* is used throughout this manuscript (Gottsch et al., 2009).

The chub mackerel, *Scomber japonicus*, is a coastal pelagic fish, belonging to the order Perciformes and family Scombridae. It is an important commercial and recreational fish throughout the tropical and temperate waters of the world, and is widely distributed in the waters off Korea, China, Japan, and California (USA) (Collette, 2003; Hwang and Lee, 2005). Due to unreliable and unpredictable wild catches, aquaculture of the chub mackerel commenced recently in southwestern Japan using young or adult fish caught from the wild (Matsuyama et al., 2005; Mendiola et al., 2008). The wild caught adult chub mackerels reared in sea pens and outdoor tanks undergo normal spermatogenesis and vitellogenesis (Matsuyama et al., 2005). This experimental system facilitates fish sampling at different gonadal stages to elucidate the role of key neuroendocrine hormones regulating seasonal gonadal development. However, female fish after the completion of vitellogenesis fails to undergo final oocyte maturation and ovulation during spawning season (April–June). This reproductive dysfunction in captive female chub mackerel seems to involve a lack of LH surge (Shiraishi et al., 2005).

As a first step to ascertain the involvement of kisspeptins in the seasonal gonadal development of chub mackerel, we characterized *Kiss1* and *Kiss2* cDNAs, and studied their distribution in different tissues. Further, mRNA expression profiles of *Kiss1* and *Kiss2* in the brain, pituitary, and gonads of adult fish during different gonadal stages were analyzed using quantitative real-time PCR.

2. Materials and methods

2.1. Fish and tissue sampling

Adult chub mackerel were caught with a purse seine and reared in sea pens at a fish farm in Oita prefecture, Kyushu Island. The fish were reared under the natural day length and fed with commercial dry pellet (Higashimaru Co., Japan) twice per day. Before the start of an experimental sampling, wild caught fish stocks were reared for 6 months. Fish sampling was carried out during the months of November 2008, early March, and late April 2009, corresponding to gonadal growth periods (Shiraishi et al., 2008). In addition, the sampling was performed (August 2009) after termination of the spawning season. During each sampling period, fish were transferred and stocked in 3-ton outdoor concrete tanks at the Tsuyazaki fishery laboratory of Kyushu University. All measures were taken to prevent handling stress. The fish were maintained in tanks supplied with natural seawater and sampling was performed after 1 week of acclimatization. At each sampling point, male and female fish (n = 8–10 for each sex) were sacrificed in accordance with the guidelines for animal experiments of the Faculty of Agriculture and Graduate Course of Kyushu University and according to the laws (No. 105) and notifications (No. 6) of the Japanese Government. Body weight and gonad weight were measured to calculate gonadosomatic index (GSI = gonad weight/body weight without gonads $\times$ 100), and the midsection of each gonad from individual fish were fixed in Bouin's solution for gonadal histology.

The brains were removed following decapitation, snap-frozen in liquid nitrogen, and stored at −80 °C until total RNA extraction. The pituitary was carefully detached from the brain tissue and stored in RNAlater (Qiagen, Hilden, Germany) solution before proceeding to RNA extraction. Gonads were dissected and one portion was snap-frozen in liquid nitrogen. Similarly, different tissues and organs of male and female fish, were removed, immediately frozen in liquid nitrogen and stored at −80 °C, until later use for total RNA extraction.

2.2. Gonadal histology

After fixation, gonad samples were dehydrated in a series of ethanol solutions up to 100%, embedded in paraffin and sectioned at 5–7 μm using a Leica RM 2155 rotary microtome (Leica, Germany). Sections were stained with hematoxylin and counterstained with eosin. The stained tissues were subsequently observed under a light microscope to confirm the gonadal stage. The male fish sampled during the months of November 2008, early March, late April, and August 2009 were divided into four gonadal stages based on histological analysis: (1) Immature (2) Late spermatogenesis (3) Spermiation and (4) Post-spawning. Similarly, female fish were classified as: (1) Immature (2) Early vitellogenesis (3) Late vitellogenesis and (4) Post-spawning.

2.3. Cloning of cDNAs for Kiss1 and Kiss2, sequencing, and phylogenetic analysis

Total cellular RNA was extracted using ISOGEN (Nippon Gene, Japan), following the manufacturer's protocol. First-strand cDNAs were synthesized from 1 μg of total RNA using an oligo(dT) primer (OdT) (Sigma) and superscript III Reverse Transcriptase (Invitrogen). To isolate the 3′ ends of *Kiss1* and *Kiss2* cDNAs, two overlapping degenerate primers (DP1 and DP2) (Invitrogen) were designed from the conserved kisspeptin-10 region of the fish *Kiss1* and *Kiss2* cDNA sequences available from the GenBank. The 3′ ends of cDNAs were amplified by two rounds of PCR using degenerate primers (DP1, DP2) and an adaptor primer (AP) as forward and reverse