Expression Profiles of Kiss2, GPR54 and GnRH Receptor I mRNAs in the Early Life Stage of Nile Tilapia, *Oreochromis niloticus*

Jin Woo Park, Jung-Hyun Kim, Ye Hwa Jin and Joon Yeong Kwon†

Dept. of Aquatic Life Medical Sciences, Sunmoon University, Asan 336-708, Korea

ABSTRACT : Kisspeptin has been implicated in the process of puberty onset in various animal groups. This peptide is encoded by a gene, Kiss1 in avian and mammalian species. Contrary to these higher vertebrates, however, fish appeared to have another gene, Kiss2 that also codes for the precursor peptide of kisspeptin. To figure out biological significance of this gene during the puberty onset in fish, the expression profile of Kiss2 gene was investigated in the brain of Nile tilapia together with genes of GPR54, GnRH receptor Ⅰ (rGnRH I) and GTH subunits (LHβ and FSHβ). Expression of Kiss2 mRNA significantly increased at 2 weeks post hatch (wph) and 13 wph (P<0.05). This increase coincided with the increases of GPR54 and rGnRH I gene expression. Detection of LHβ and FSHβ subunit gene expression was possible later than 13 wph, indicating the activation of gonadotrophs in the pituitary. Data obtained from this study strongly suggest that, in addition to Kiss1 gene, Kiss2 gene is deeply associated with the onset of puberty by the activation of hypothalamus pituitary gonadal axis in Nile tilapia.

Key words : Kiss2, GPR54, rGnRH Ⅰ, LHβ, FSHβ, Puberty, Nile tilapia, *Oreochromis niloticus*

INTRODUCTION

The hypothalamic-pituitary-gonadal (HPG) axis is generally known to govern the reproductive processes in a variety of vertebrates including fish (reviewed by Zohar et al., 2010). However, the factors and mechanism that regulate HPG axis in further upstream have not yet been clearly established. Recent studies have suggested that kisspeptin and its cognate receptor, GPR54 (G protein coupled receptor 54) could have some important roles in the control of the neuroendocrine regulation of reproduction by modulating HPG axis in various vertebrate species (Oakley et al., 2009; Zohar et al., 2010).

Kisspeptin, also known as metastin, belongs to the RFamide peptide family and exists various forms such as kisspeptin-54, kisspeptin-14, kisspeptin-13, and kisspeptin-10 in mammals (Bilban et al., 2004) depending on the way of preprohormone processing (Ohtaki et al., 2001). GPR54 shows high affinity with kisspeptin-10 which is common in the C-terminal of all kisspeptin forms (Kotani et al., 2001). Originally, Kiss1 was identified as a metastasis suppressor gene in humans (Lee et al., 1996), but is now recognized as a crucial gene that control reproduction. Various functions of kisspeptin/GPR54 system have been suggested in relation to the reproduction of vertebrates (Table 1). The majority of the suggested functions are largely involved with the activation of HPG axis and stimulation of GTH (gonadotropin) secretion. Because of this, kisspeptin/GPR54 system is being intensively studied as a potential activator of HPG axis during the onset of puberty in vertebrates (reviewed by Kauffman et al., 2007).

Kisspeptin is encoded by a gene, Kiss1 in avian and mammalian species. Contrary to these higher vertebrates, fish appeared to have another gene, Kiss2 that also codes for the precursor peptide of kisspeptin. Kiss2 gene have already been identified in several teleosts such as medaka, *Oryzias latipes* and zebrafish, *Danio rerio* (Kitahashi et al., 2009), European seabass, *Dicentrarchus labrax* L. (Felip et al., 2009), goldfish, *Carassius auratus* (Li et al., 2009),

† Corresponding author: Joon Yeong Kwon, Dept. of Aquatic Life Medical Sciences, Sunmoon University, Asan 336-708, Korea. Tel: +82-41-530-2284, Fax: +82-41-530-2917, E-mail: jykwon@sunmoon.ac.kr

Table 1. Suggested functions of kisspeptin in relation to the reproductive processes of vertebrates

Animals	Suggested functions	Reference
Human	Stimulates LH, FSH and testosterone secretion	Dhillo et al., 2007
Mouse	Stimulates LH and FSH secretion	Messager et al., 2005
Rat	Stimulates LH and FSH secretion	Patterson et al., 2006
Goldfish	Induces GH, PRL, LH secretion	Yang et al., 2010
Medaka	Sexual dimorphism in kisspeptin neurons	Kanda et al., 2008
Seabass	Induces GTH secretion	Felip et al., 2009
Zebrafish	Potent activator for GTH secretion	Kitahashi et al., 2009

LH: luteinizing hormone, FSH: follicle stimulating hormone, GH: growth hormone, GTH: gonadotropin, PRL: prolactin.

chub mackerel, *Scomber japonicus* (Selvaraj et al., 2010). In zebrafish (Kitahashi et al., 2009) and mackerel (Selvaraj et al., 2010), Kiss1 and Kiss2 genes were differentially expressed in the brain and peripheral tissues (Kitahashi et al. 2009), implying some possible differences in their actions on LH and FSH secretion/expression with regard to the reproduction and puberty onset.

Nile tilapia, *Oreochromis niloticus*, an important aquaculture species worldwide, is a mouse-brooding and multiple spawning fish species which shows high spawning frequency. Such high frequency is not favorable for the culture of this species. Artificial control of puberty onset could greatly improve the productivity of culture in this fish. However, development of puberty control technique requires further understanding of the mechanism of puberty onset. Involvement of GPR54 to the onset of puberty was suggested several years ago (Martinez-Chavez et al., 2008), but the role of kisspeptin/GPR54 system in this species is far from clear.

In this study, we examined gene expression profiles of Kiss2, GPR54, GnRH receptor Ⅰ (rGnRH Ⅰ) and GTH subunits (LHβ and FSHβ) during the early life stage in Nile tilapia to improve our understanding for the mechanism of puberty onset.

MATERIALS AND METHODS

Hatchlings of Nile tilapia, *O. niloticus* were produced in fish rearing facilities in Sunmoon University (Asan, Chung Nam, Korea). They were reared in a closed recirculating culture system with water temperature at 27±1℃ under natural photoperiod and fed twice a day. Fish were sampled every week at noon from 1 day post hatch (0 week) to 24 weeks post hatch (wph) during which sex differentiation and puberty onset were expected to be taken place. The number of fish sampled and pooled varied depending on the size of the fish (Fig. 1, Table 2).

Sampled tissues were homogenized in 1 ㎖ TRI-REAGENT (Molecular Research Center) per 100 ㎎ of tissue. The concentrations of RNA samples were measured by a spectrophotometer (GeneQuant™ *pro* RNA/DNA Calculator, Amersham Pharmacia Biotech, Uppsala, Sweden) and adjusted to 1 ㎍/㎕. All reagents used in cDNA synthesis were purchased from Promega (USA). Each total RNA (1 ㎍) sample was treated with DNase Ⅰ (RQ1 RNase-free DNase) to remove genomic DNA and then, reverse transcribed according to manufacturer's protocol

Table 2. The number of samples and fish pooled in a sample at each stage

Stage (wph)	No. of fish in a sample (Regions of sampling)	No. of sample (n)
0	20 (heads)	4
1	15 (heads)	4
2-3	10 (heads)	4
4	8 (heads)	4
5-6	5 (heads)	4
7	4 (heads)	4
8	3 (heads)	4
9-10	2 (brains)	4
11-24	1 (brain)	4

wph: weeks post hatch.

using Oligo $(dT)_{15}$ primer, 10 mM dNTP mix, M-MLV (Moloney murine leukemia virus) reverse transcriptase and provided buffer.

The resultant cDNAs were used as templates for quantitative real-time PCR (qRT-PCR) to study the expression profiles of Kiss2, GPR54, rGnRH I, LHβ subunit and FSHβ subunit genes. All qRT-PCR reactions containing 0.5 $\mu\ell$ of each primer (10 pmol/$\mu\ell$), 2 $\mu\ell$ cDNA, 10 $\mu\ell$ QuantiMix SYBR (PhileKorea, Korea) and 7 $\mu\ell$ of Nuclease-free water in a final volume of 20 $\mu\ell$, were performed using the Eco™ Real-Time PCR System (illumina®, USA). For normalization, the expression of β-actin was also analyzed. The conditions for each PCR reaction were as follows: initial denaturation at 95℃ for 15 minutes, 45 amplification cycles including denaturation at 95℃ for 15 seconds, annealing at 60℃ for 15 seconds and elongation at 72℃ for 30 seconds. To confirm the specificity, a melting curve analysis was performed at the end of PCR amplifications. The expression levels of each genes were reported as threshold cycle (Ct) values. Relative expression of each target gene was determined by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

Primers for Kiss2, LHβ, FSHβ and β-actin genes were designed using Primer3 Ver. 0.4.0 program (http://frodo.wi.mit.edu/) on the basis of Kiss2, LHβ, FSHβ and β-actin gene sequences of Nile tilapia obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Primers for tilapia GPR54 and rGnRHⅠ gene were synthesized based on the primer sequences published by Martinez-Chavez et al. (2008). GenBank accession numbers and sequences of primers for each gene are shown in Table 3.

Results were expressed as mean±SEM. One-way ANOVA followed by Duncan's multiple range test was performed to determine statistical significances in the changes of Kiss2, GPR54, rGnRHⅠ, LHβ and FSHβ mRNA expressions during the early life stage (SPSS v18, $P<0.05$).

RESULTS

No significant reduction of fish number by mortality was observed during the period of sampling. Fish grew slowly from 0.020±0.003 g at hatching to 1.973±0.541 g at 12 wph, showing small weight gain (less than 2 g per individual). However, the weight gain in the following 12 weeks was more than 45 g/individual, implying that the fish were on the course of pubertal growth spurt (Fig. 1).

Kiss2 mRNA expression increased significantly at 2 wph but remained low without any significant changes

Table 3. Sequences of primers used for quantitative real-time PCR

Gene		Primer sequence	Product size (bp)	Accession No.
Kiss2	F	5'-CTACTGGCTTTGGCTGTGGT-3'	112	JN565693.1
	R	5'-CTGCTCCTGTTGCATGTGTT-3'		
GPR54	F	5'-ATGCCTGGCTGGTCCCTCTGTTCT-3'	136	AB162143.1
	R	5'-GGCGGCCAGGTTTGCTATGTA-3'		
GnRHr Ⅰ	F	5'-GTGGCTTGCCGGAGACTTTG-3'	123	AB111356
	R	5'-AGAGGGTTGAGGATGGCTGACT-3'		
LHβ	F	5'-GCATGTGTGCACATACCGGG-3'	106	AY294016.1
	R	5'-GTGGCAACTCAAAGCCACGG-3'		
FSHβ	F	5'-GGCTTCGTCGACACCACCAT-3'	148	AY294015.1
	R	5'-TGAAACCCCGTGGACATTGC-3'		
β-actin	F	5'-AGCATCCCGTCCTGCTCACA-3'	121	EU887951.1
	R	5'-AGCACAGCCTGGATGGCAAC-3'		

F: forward, R: reverse.

until 12 wph. The level increased significantly at 13 wph and maintained high until 24 wph (P<0.05)(Fig. 2). The expression profile of GPR54 mRNA was almost identical to that of Kiss2, showing significant increase at 2 and 13 wph (P<0.05)(Fig. 3). The pattern of rGnRH Ⅰ mRNA expression was also similar to those of Kiss2 and GPR54 showing significant increases at 2, 11, 13 and 21 wph (P<0.05)(Fig. 4).

The level of LHβ subunit mRNA was low until 16 wph and increased sharply at 17 wph (P<0.05). Since then, the expression level maintained high throughout the remaining period (Fig. 5). Expression of FSHβ subunit mRNA was detected from 13 wph and increased significantly at 21 wph (Fig. 6).

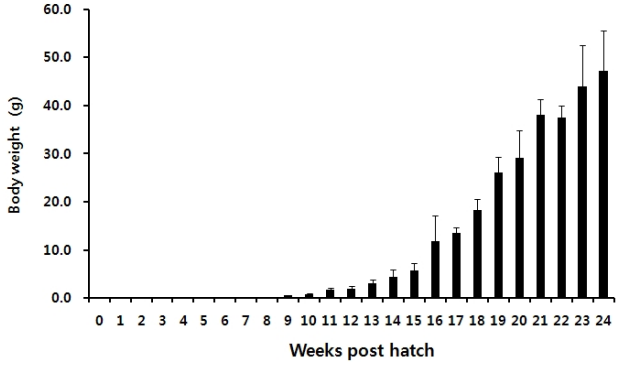

Fig. 1. The size (body weight) of the fish sampled from hatching to 24 weeks post hatch. Data are expressed as mean±SEM.

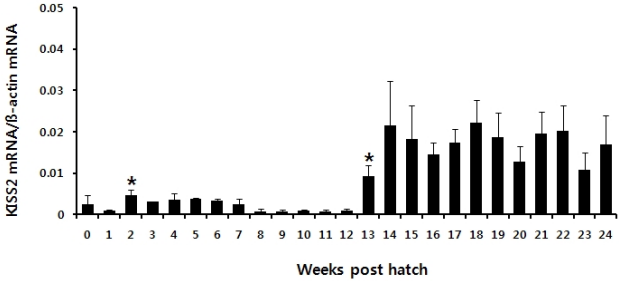

Fig. 2. Expression profile of Kiss2 gene in Nile tilapia from hatching to 24 weeks post hatch. The expression of Kiss2 gene and β-actin gene (as a control) were examined by using real-time PCR. Data are expressed as mean± SEM. * indicates significant difference from the respective previous week (P<0.05).

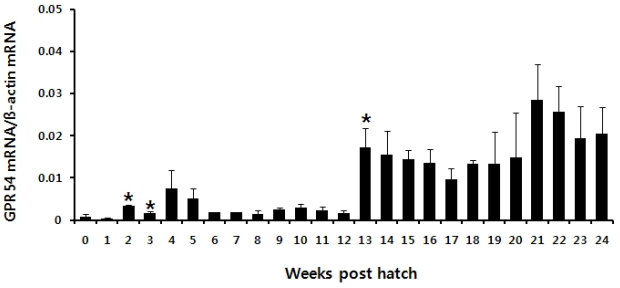

Fig. 3. Expression profile of GPR54 gene in Nile tilapia from hatching to 24 weeks post hatch. The expression of GPR54 gene and β-actin gene (as a control) were examined by using real-time PCR. Data are expressed as mean± SEM. * indicates significant difference from the respective previous week (P<0.05).

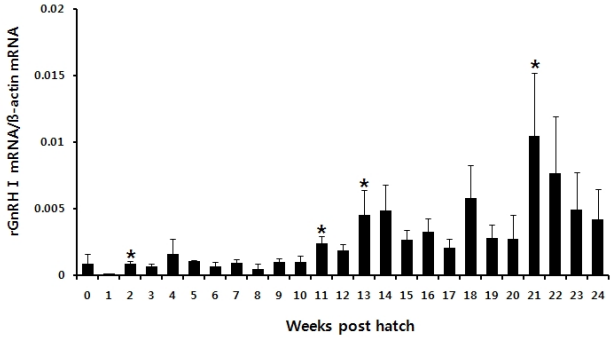

Fig. 4. Expression profile of rGnRH Ⅰ gene in Nile tilapia from hatching to 24 weeks post hatch. The expression of rGnRH Ⅰ gene and β-actin gene (as a control) were examined by using real-time PCR. Data are expressed as mean±SEM. * indicates significant difference from the respective previous week (P<0.05).

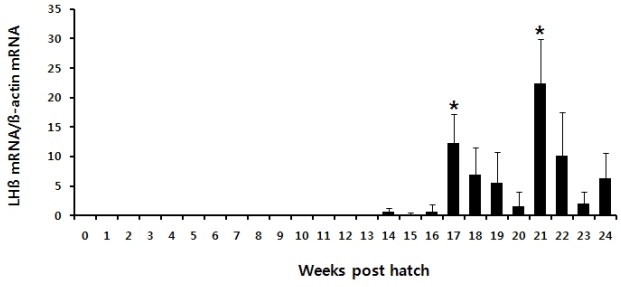

Fig. 5. Expression profile of LHβ subunit gene in Nile tilapia from hatching to 24 weeks post hatch. The expression of LHβ subunit gene and β-actin gene (as a control) were examined by using real-time PCR. Data are expressed as mean±SEM. * indicates significant difference from the respective previous week (P<0.05).

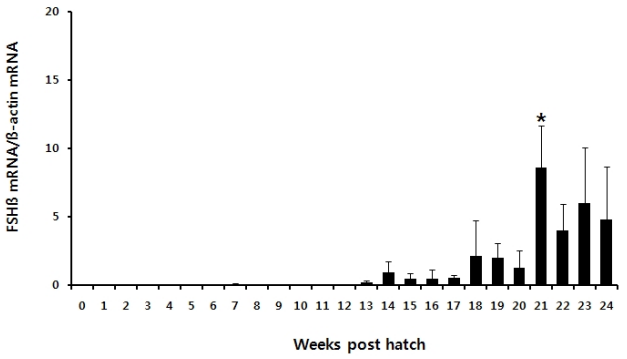

Fig. 6. Expression profile of FSHβ subunit gene in Nile tilapia from hatching to 24 weeks post hatch. The expression of FSHβ subunit gene and β-actin gene (as a control) were examined by using real-time PCR. Data are expressed as mean±SEM. * indicates significant difference from the respective previous week (P<0.05).

DISCUSSION

Gene expression profiles of Kiss2, GPR54, rGnRH I and GTH subunits (LHβ and FSHβ) revealed a putative timing of puberty onset at around 13 wph and a close relationship between Kiss2 and puberty onset in Nile tilapia.

The timing of puberty onset in fish is essentially species-specific, but it can also be strain-specific within a species. Red Nile tilapia is a color variation of Nile tilapia sharing all characteristics of the species except body color. Puberty onset in red Nile tilapia had been suggested at around 9-11 wph in female and 11-13 wph in male based on histological observations and the significant increases of GPR54 and rGnRH I mRNAs (Martinez-Charvez et al., 2008). Results from the present study showed significant increases of these two genes at 13 wph, implying the onset of puberty. The differences between red and ordinary Nile tilapia in terms of the initiation of puberty could simply be attributed to the difference of strains. Other possible causative of the difference could be related to the difference of growth rates in two different experimental system. Indeed, red tilapia in the study of Martinez-Chavez et al. (2008) grew about two to three weeks faster than the fish in the present study did (Fig. 1). Possibly because of this, the timing of the puberty onset in this study was two weeks behind the red tilapia. Supportingly, in salmon, individuals that enter puberty early are commonly the larger individuals within a population (Skilbrei, 1989).

Growth spurt during puberty is a general knowledge in human biology. Growth increase has also been demonstrated during the early stage of pubertal development in fish (Skilbrei, 1989; Kadri et al., 1996). The growth of body weight in Nile tilapia in this study increased markedly before and after 13 wph (Fig. 1) coinciding with the presumptive timing of puberty onset. This implies that the fish in this study were on the course of pubertal growth spurt from 13 wph.

Kiss1 is known to play a key role in activating HPG axis in mammals (reviewed by Zohar et al., 2010). In fish, however, expression of Kiss2 gene have been reported in the brains and gonads of seabass, medaka and zebrafish. Injection of synthetic Kiss2 peptide to seabass was found to be more potent than synthetic Kiss1 peptide in inducing FSH and LH release (Felip et al., 2009). Furthermore, synthetic Kiss2 peptide were shown to be able to activate all forms of GPR54, indicating that Kiss1 and Kiss2 peptides could share common receptors (Lee et al., 2009) even though both peptides may have their own receptors, namely GPR54-1 and GPR54-2.

Kiss2, GPR54 and rGnRH I mRNAs were concomitantly increased during the early life stage of Nile tilapia in the present study, indicating that these genes work in concert to regulate crucial reproductive events (such as sex differentiation and puberty onset) in fish at early stage of their life. In agreement with this finding, concomitant increases of GPR54 and GnRH mRNAs at 26 dph in a teleost, cobia, *Rachycentron canadum* (Mohamed et al., 2007), the number of rGnRH I and Kiss2 neurons in the brains of female red seabream, *Pagrus major* in the first spawning period (Shimizu et al., 2012), and the expression of GPR54 and rGnRH Ⅰ genes in red Nile tilapia at 11 wph (Martinez-Chavez et al., 2008) have been reported. In addition, Kitahashi et al.

(2009) also reported the increase of Kiss2 gene expression in the brain of medaka and zebrafish during puberty.

It has been generally recognized that in Nile tilapia sex differentiation occurs about 10-35 days post fertilization (Nakamura & Nagahama, 1985, 1989; Kwon et al., 2001, 2006). The first significant increases of Kiss2, GPR54 and rGnRH Ⅰ mRNAs coincided with the suggested sex differentiation period (2-4 wph) in this species. Similarly, significant increases of GPR54 and GnRH mRNAs during early developmental stages (2-3 dph) were also observed in cobia (Mohamed et al., 2007). In support of this, Shi et al. (2010) found that expression of Kiss2 gene was suppressed during female to male sex reversal by the injection of 17α-methyltestosterone in orange-spotted grouper, *Epinephelus coioides*. These data are suggestive of some possible involvement of Kiss2 in the process of gonadal sex differentiation. However, the exact role of Kiss2 during the period of sex differentiation requires further studies.

Detection of the expression of LHβ and FSHβ subunit genes was possible from 13 wph when Kiss2, GPR54 and rGnRH I mRNA increased significantly in this study. Expression of GTH subunit genes are indicative of pituitary activation, and subsequent gonadal activation and development. Supporting this, in European male seabass, expressions of LHβ and FSHβ genes in the pituitary were significantly up-regulated in the tentative gonadal development period (Rodríguez et al., 2005). In addition, expressions of three GnRH mRNAs increased concomitantly with the expressions of GnRH receptor, FSHβ, FSH receptor and LH receptor genes during early development of gilthead seabream, *Sparus aurata* (Wong et al. 2004). Since 13 wph in the present study, the expression of Kiss2, GPR54 and rGnRH I mRNA maintained much higher levels compared with the levels earlier than 13 wph. During which Kiss2, GPR54 and rGnRH I mRNA maintained higher, the expression of LHβ and FSHβ subunit genes increased and maintained high. The results from the present study clearly suggest that Kiss2 signal wakes up the GTH producing potentials of pituitary, possibly via GnRH neurons with GPR54 in the hypothalamus, leading fish to the gate of puberty.

In conclusion, data obtained from this study strongly suggest that, in addition to Kiss1 gene, Kiss2 gene is deeply associated with the onset of puberty by the activation of HPG axis in Nile tilapia. Furthermore, the data also suggest that Kiss2 signal might be involved in the process of gonadal differentiation in fish.

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