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Polymorphisms and DNA methylation level in the CpG site of the *GHR1* gene associated with mRNA expression, growth traits and hormone level of half-smooth tongue sole (*Cynoglossus semilaevis*)

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Abstract The objectives of the present study were to estimate the *GHR1* gene mutations and methylation status of CpGs, and whether those mutations and methylation were involved in the regulation of *GHR1* gene expression, hormone level and growth traits in half-smooth tongue sole (*Cynoglossus semilaevis*). Identification of single-nucleotide polymorphisms was performed on 43 male fish. Through polymerase chain reaction-single-strand conformation polymorphism and sequencing, two SNPs were found. SNP1 [c.G1357A (p.Val376Ile)] creating one CpG site located in exon 8 was named L1 locus, and SNP2 (c.G1479A) located in exon 9 was named L2 locus. Individuals were divided into three genotypes, AA, AG and GG according to L1 locus (GG genotype had one more CpG site because of the mutation), and into two genotypes, AA- and GG-based on L2 locus. The results showed that only L1 locus was significantly associated with body weight ($P < 0.01$), gonad weight ($P \leq 0.05$), triiodothyronine (T3) level ($P \leq 0.05$) and mRNA expression ($P < 0.01$). At L1 locus, newly created CpG site in GG genotype was highly methylated (93.3 %), while there was no difference of methylation level in the other two CpG sites among three genotypes. AA genotype and AG genotype having higher T3 level were significantly different ($P \leq 0.05$) from GG genotype. There were significant differences among body weights of AA, AG and GG genotypes ($P < 0.01$). Gonad weights of AA genotype and AG genotype were significantly lower than GG genotype. The *GHR1* mRNA expression of GG genotype was significantly lower than AA and AG genotypes ($P < 0.01$). These implied that mutations and methylation status of *GHR1* gene might influence the hormone level, growth traits and gene expression in male half-smooth tongue sole and the L1 locus could be regarded as a potential candidate genetic and epigenetic marker in half-smooth tongue sole selection.

Keywords *GHR* gene · SNPs · DNA methylation · Gene expression · Growth traits · Half-smooth tongue sole (*Cynoglossus semilaevis*)

Introduction

The biological effects of growth hormone (GH) concern in stimulating several anabolic process, such

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as cell proliferation, skeletal growth and protein synthesis (Burton et al. 1994). The action of GH is initiated by its binding to GH receptor (GHR) on the cell membrane of various target tissues. The combination of GH with GHR triggers a phosphorylation cascade for signaling and gene expression, which results in biological actions of GH. The number, structure, normal function and the change of bases sequence of GHR may affect the physiological function of GH. GH effects on *IGF-I* expression may be related with higher contents of truncated GHR1 (Fuentes et al. 2013). Some molecular markers of *GHR* gene related to body weight, total length and body length have been screened in *Paralichthys olivaceus* (Ni et al. 2008) and *Cyprinus carpio* var. Jian (Tao et al. 2011). *GH* gene polymorphism was significantly associated with *GHR* and *IGF-I* gene expression in Nile tilapia (Tanamati et al. 2015). *GHR* has a close relationship with *GH* and can be an indicator of growth status in fish.

Up to now, *GHR* gene has been reported in many vertebrate, including mammals and fish. It is surprising that there are only one type of GHR in mammals, but there are two different isoforms in fish, such as gilthead sea bream (*Sparus aurata*; Saera-Vila et al. 2005, 2007), sea bream (*Acanthopagrus schlegelii*; Jiao et al. 2006), Japanese eel (*Anguilla japonica*; Ozaki et al. 2006), orange-spotted grouper (*Epinephelus coioides*; Li et al. 2007), Nile tilapia (*Oreochromis niloticus*; Ma et al. 2007) and half-smooth tongue sole (*Cynoglossus semilaevis*; Ma et al. 2012a). GHRs belong to the class I cytokine receptor superfamily, which have three domains: ligand-binding domain, transmembrane domain and intracellular domain. Highly conserved regions (Box1 and Box2), which are important to signal transduction of the receptors, are found in the intracellular domain (Kopchick and Andry 2000). Any mutation of the amino acid residues within the boxes can result in inactivation of GH. GHR1 and GHR2 possess features that are distinctly different from each other, and they contain different numbers of extracellular and intracellular cysteine residues (Zhang et al. 2006; Li et al. 2007). Besides this, *GHR1* gene and *GHR2* gene also have different tissue distributions and even differential gene expression levels on the basis of age, season, environment, hormone level and nutritional condition (Tao et al. 2011). Tissue distributions of the *GHR* mRNA of fish are detected in brain, kidney, stomach, gonad etc., especially in liver and muscle (Ozaki et al. 2006; Tao et al. 2011). Calduch-Giner et al. (2003) had reported that *GHR* mRNA expression of gilthead sea bream (*Sparus aurata*) exhibited a higher level in summer than in spring. In *Megalobrama amblycephala*, *GHR1* had a similar expression pattern to *GHR2* during the developmental stages ($P < 0.05$; Zeng et al. 2014). Saera-Vila et al. (2009) reported a differential expression of the two types of *GHRs* in gilthead sea bream. Structural differences and expression differences of *GHR1* and *GHR2* indicated that they may have different biological functions. GHR1 had the similar function with GHR in mammals. The two isoforms of GHRs were generated by alternative splicing, but their specific functions are still unclear now.

Mutations in *GHR* gene sequence may influence the expression of *GHR* gene, combination capacity of GH or the conformation of the binding GH. These may eventually affect the growth of animals. Research showed that *GHR* gene expression reduced following a three-week fast, the binding rate of GH to GHR decreased, and the growth-promoting function of GH was weaken and then slowed down fish growth (Fukada et al. 2004). But in tilapia, *GHR* expression in muscle increased obviously during fasting and then declined below control levels upon re-feeding (Fox et al. 2010). These results indicated that *GHR* is sensitive to nutritional status and then affect growth condition. *GHR* gene is considered a potential and functional candidate gene for selecting high-growth animals. Currently, studies concerning in *GHR* polymorphisms and its association with different traits are mainly focused on livestock and poultry. Zhou and Jiang (2006) reported that a SNP in bovine *GHR* exon 8, causing the substitution of phenylalanine with tyrosine (F279Y), showed significant effects on milk composition. It was shown that some productive traits of cattle, for example, milk yield, growth performance and carcass traits were correlated with the polymorphisms of the *GHR* gene (Maj et al. 2004). Single-nucleotide polymorphisms (SNPs) are one of the suitable molecular markers for fine candidate genes association studies aimed at identifying alleles potentially affecting important traits. Because of its representativeness, genetic stability, etc., SNP markers have been used to screen candidate genes for growth in some aquatic species, including Asian seabass (*Lates calcarifer*; Xu et al. 2006), *Penaeus vannamei and Penaeus monodon* (Glenn et al. 2005). But it is still a relatively new tool in aquaculture. It was also reported

that SNPs have an impact on gene expression and the function of proteins. It is anticipated that SNPs markers will contribute greatly to studies on aquatic animal population genetics, molecular breeding as well as evolutionary biology.

Changes in DNA structure can regulate gene expression in some extent. Mutations in CpG rich areas in exons included G → T transition, as well as C → T and G → A transition. These mutations have always affected the rate of cytosine methylation (a kind of epigenetic modification), and cytosine methylation is a restraining factor of gene activation status. It was shown in mammals that methylation in a CpG-rich region of the fatty acid desaturases2 could modulate its expression contributing to the pathology of hyperhomocysteinemia (Devlin et al. 2005). Also, there were few relative studies in the aquatic species.

Half-smooth tongue sole (*C. semilaevis*) mainly distributes in the East Asia. Because of its delicious taste, high nutrient value and many other advantages, half-smooth tongue sole has became one of the most important culture species in China. The male individuals are much smaller than female. In the process of fast-grown family selection, emphasis should be put on the selection of male fish, to improve the quality of male parent. This is for cultivating the offspring with better performance. There is a need for building high-performing breeding programs, with the assistance of molecular marker techniques. Selected individuals with excellent growth traits can benefit both production and scientific research.

GHR1 and *GHR2* in *C. semilaevis* had been cloned by Ma et al. (2012a). Whole-genome sequence of half-smooth tongue sole had been published by Chen et al. (2014). Objectives of the present study were to identify polymorphisms and DNA methylation status in half-smooth tongue sole *GHR1* gene and to determine whether those mutations and methylation level had regulatory effects on growth performance and gene expression in these animals, which may contribute to the breeding of this species of fish.

Materials and methods

Animals and data

Forty-three adult male half-smooth tongue sole of the same age and size were obtained from a local fish farmer (mean length and weight were 225.39 g and 32.50 cm). They were reared in commercial fish ponds for twenty months, under the controlled conditions (20 ± 0.5 °C; 14:10-h light/dark cycle; $O_2 \geq 4$ ng/ml), and fed on a prepared diet. After one or 2 days of temporary rearing (unfed), the fish were lightly anesthetized and sample of blood were obtained from the caudal vein. Before dissected, body mass and body lengths were measured, and during dissection, weights without viscera, gonad weights and liver weights were measured. Then different tissues were immediately frozen in liquid nitrogen and kept at −80 °C until use. HSI or GSI of each fish was calculated as follows:

$$\text{Hepatosomatic or gonadosomatic index} = [\text{liver or gonad weight}/(\text{body mass} - \text{viscera weight})] \times 100\ \%$$

Steroid radioimmunoassay

After 8-h storage at 4 °C, blood samples were centrifuged at 12,000 rpm for 10 min and their serum were used for the test of triiodothyronine (T3), tetraiodothyronine (T4) and IGF-I by radioimmunoassay, according to the protocol provided by Ding et al. (2012).

DNA extraction

Genomic DNA was isolated using a phenol–chloroform extraction procedure (Ma et al. 2012b) from the muscle tissues. The quality and concentration of DNA were assessed by Ultramicro Nucleic Acid and Protein Analyzer (BD-1000), followed by the dilution to about 150 ng/μl, and their integrity was evaluated by agarose gel electrophoresis.

PCR-SSCP analysis

Primers were designed using Oligo 6 software to amplify the exons of *GHR1* gene (Table 1) of the experimental half-smooth tongue sole, according to the cDNA sequence (GenBank Accession No. FJ608664), except the primers of exons 6 and 7, whose sequences were less than 100 bp. Among these primers, Primer 1 and Primer 2 were used to amplify exon 1, Primer 7 and Primer 8 were used to amplify exon 8, and primers

Table 1 Primers used for amplification exons of *GHR1* gene and quantitative PCR

Primer name	Sequence (5′–3′)	Annealing temp. (°C)	Fragment length (bp)	Site (bp)	
Primer1	GCGGGGGCTGTTCAGGAT (F)	61.9	157	3–159	(exon 1)
	GCACCAACAGGCACGGAAG (R)				
Primer2	TCCGTGCCTGTTGGTGCT (F)	61.9	205	143–347	
	GATGACGCCCTTTGGTCCC (R)				
Primer3	TCACTCTTGAGCCTCACAT (F)	55.0	122	350–471	(exon 2)
	AAGGTAGAAGAATCTGAGTG (R)				
Primer4	GTCTCCAAACAGCCAGTG (F)	57.0	170	480–649	(exon 3)
	CTATATCTTCCACACTGAAAC (R)				
Primer5	GTCCCGACCCTCCAGTGAA (F)	61.3	175	653–827	(exon 4)
	GCTTCCCACTTTGTTGTATTTC (R)				
Primer6	CTGGAGATGCAACCACAA (F)	55.4	154	829–982	(exon 5)
	CTGTGCTTTCAACCTCACT (R)				
Primer7	AAAGGGAAGTTGGAAGAG (F)	57.6	133	1144–1276	(exon 8)
	AATCAGTGTCCTCAGCAT (R)				
Primer8	GAGGACACTGATTCAGGG (F)	57.6	113	1264–1376	
	ATGGTGTTGGCACAGCCTA (R)				
Primer9	TAGCTTCCCTGATGATGAC (F)	55.4	244	1380–1623	
	CATAACATTGCTGACCTGG (R)				
Primer10	CCCAGGTCAGCAATGTTA (F)	55.4	263	1604–1866	(exon 9)
	TATGGTTTGGTAGCCCTC (R)				
Primer11	AGGGCTACCAAACCATACA (F)	55.4	240	1850–2089	
	GCATACCAGATAGGGACTT (R)				
Primer12	CCTATCTGGTATGCCTGT (F)	55.4	302	2076–2377	
	TTTACCATCCGCAGCCTA (R)				
Real-time primer	TCTGTTGCCACCAGTTCC (F)	57	183		
	TCCTCAGCATCCACCTCA (R)				
β-Actin primer	GTAGGTGATGAAGCCCAGAGCA (F)	57	157		
	CTGGGTCATCTTCTCCCTGT (R)				

9–12 were used to amplify exon 9. DNA was amplified in 25-μl volume system, containing 0.2 mM each dNTP, 2.5 μl 10 × PCR buffer, 0.20 mM primers and 0.5 U EasyTaq DNA polymerase (TransGen Biotech, China) in five steps: (1) initial denaturation of the double strand at 94 °C for 5 min, (2) denaturation at 94 °C for 30 s, (3) annealing of the primer at Tm for 30 s, (4) extension at 72 °C for 35 s and (5) a final extension at 72 °C for 10 min. Steps 2, 3 and 4, corresponding to one cycle, were repeated 40 times. DNA fragments were evaluated on 2 % agarose gels.

Five-microliter PCR products of *GHR1* gene from each individual were mixed with 8 μl denaturing buffer, (containing 98 % formamide, 0.09 % xylene cyanole FF and 0.09 % bromophenol blue per 20 ml) and then denatured at 98 °C for 10 min and a rapid chill on ice for 10 min soon afterward. The whole denatured products were loaded on 8–12 % acrylamide/bisacrylamide (aci:bis = 29:1) gel at 120 V for 10–12 h, based on the lengths of amplified products [taking 100-bp product for an example, the loading system containing 12 ml 30 % PAGE (12 %), 3 ml 10 × TBE, 15 ml ddH_2O, 0.02 g APS and 12 μl TEMED], and the gels were stained using the silver staining method. PCR products of different genotype samples were purified with TIANgel Midi Purification Kit (TIANGEN, China) and then inserted into the pEASY-T1 vectors (TransGen Biotech, Beijing) and transferred into Escherichia coli Trans1-T1. Recombinants were sequenced by the BGI Company (Beijing, China).

Quantitative real-time PCR

Total RNA was extracted from liver tissues from three different individuals of each genotype based on exon 8 with RNAiso reagent (TakaRa, Japan). Then genomic DNA elimination reaction and reverse-transcription reaction were performed with PrimeScript RT reagent Kit (TaKaRa, Japan) under the manufacturer's instruction.

The expression level of *GHR1* gene was quantified using SYBR Premix Ex Taq (TaKaRa, Japan) on the Realplex PCR machine. Quantitative realtime qRT-PCR primers of *GHR1* gene were designed by Primer 5 software (Table 1). The amplifications were carried out in a volume of 20 μl containing 10 μl SYBR, 0.4 μl of each primer and 2 μl diluted cDNA. Cycling conditions were 95 °C for 2 min; 40 cycles at 95 °C for 15 s, 57 °C for 15 s and 72 °C for 20 s; 95 °C for 15 s; 57 °C for 15 s; 57–95 °C for 20 min; and 95 °C for 15 s. The experiment was performed in three replicates. β-Actin (GenBank Accession No. AY116536), used as internal reference, was amplified under the same reaction conditions with *GHR1* gene. Relative expression was normalized by β-actin (calculation method: $2^{-\Delta\Delta C_t}$, where $\Delta\Delta C_t = \{[C_t(\text{GHR1}) - C_t(\beta - \text{actin})] - [C_t(\text{GHR1})\ \text{reference} - C_t(\beta - \text{actin})\text{reference}]\}$). *GHR1* gene mRNA expression level was compared using paired *t* test. A probability level of $P \leq 0.05$ was considered statistically significant.

Methylation analysis

High-purity DNA extraction from liver tissue of three individuals and bisulfite treatment were performed using Marine Animals DNA Kit (CWBIO) and Methylamp™ DNA Modification Kit (QIAGEN), respectively, under the manufacturer's instructions. The individuals, consistent with the ones performed RNA isolation, were chosen from each genotype based on exon 8. The treated DNA was stored at −20 °C until analysis.

Specific primers, generating a 223-bp targeted PCR product, were as follows: 5′-AGAGTTGAATTT GATTTTTAG-3′ (forward) and 5′-CTAATAATAT TAACACAACCTA-3′ (reverse). PCR was carried out in a 25-μl volume system, consisting of 0.125 μl EpiTaq HS (TaKaRa), 2.5 μl EpiTaq PCR buffer (Mg^{2+} free), 3 μl dNTP mixture, 3 μl template (<100 ng) and 1 μl of each primer. Amplification conditions were as follows: 94 °C for 5 min; 40 cycles of 94 °C for 30 s, 49 °C for 30 s and 72 °C for 30 s; and 72 °C for 5 min.

The amplified PCR products were then identified on 2 % agarose gels and sequenced as the method described above. At least ten random clones of each individual were sent to be sequenced as to calculate the rates of methylaion.

Statistical analysis

The association between mutations and measured traits or gene expression was analyzed by one-way ANOVA, using Stat View software version 9.0 (SAS Institute Inc., Cary, NC, USA). Difference among means of different genotypes was assessed using Duncan multiple-range test. Statistical significance was considered as $P \leq 0.05$.

Results

Polymorphisms within exons of *GHR1* gene and genotyping

The A and G alleles of the genomic *GHR1* gene were identified based on the amplification using primers 8 and 9. Two loci named L1 locus (c.G1357A; Fig. 1a) and L2 locus (c.G1479A; Fig. 1b) were a non-synonymous mutation, inducing an amino acid change from Val (376) to Ile (376) and a synonymous mutation, respectively. The genotypes were defined as GG, AA and AG at L1 locus (Fig. 1c, d) and GG and AA at L2 locus (Fig. 1e, f).

Correlation between mutation locus and measured traits

The association between the loci of *GHR1* gene and the measured traits of half-smooth tongue sole was shown in Table 2. Statistic showed that L1 locus had significant associations with T3, gonad weights ($P \leq 0.05$) and weights ($P < 0.01$), but had no significant associations with T4 and IGF-1. Multiple comparison analysis indicated that GG genotype had lower T3 level [2.03 ± 0.4069 (ng/ml)], but higher gonad weights (1.71 ± 0.2780 g) than AA (2.45 ± 0.3152 ng/ml; 1.02 ± 0.2154 g) and AG genotypes (2.93 ± 0.1191 ng/ml; 0.89 ± 0.0814 g; Fig. 2a, b). The result also showed that there was significant difference among

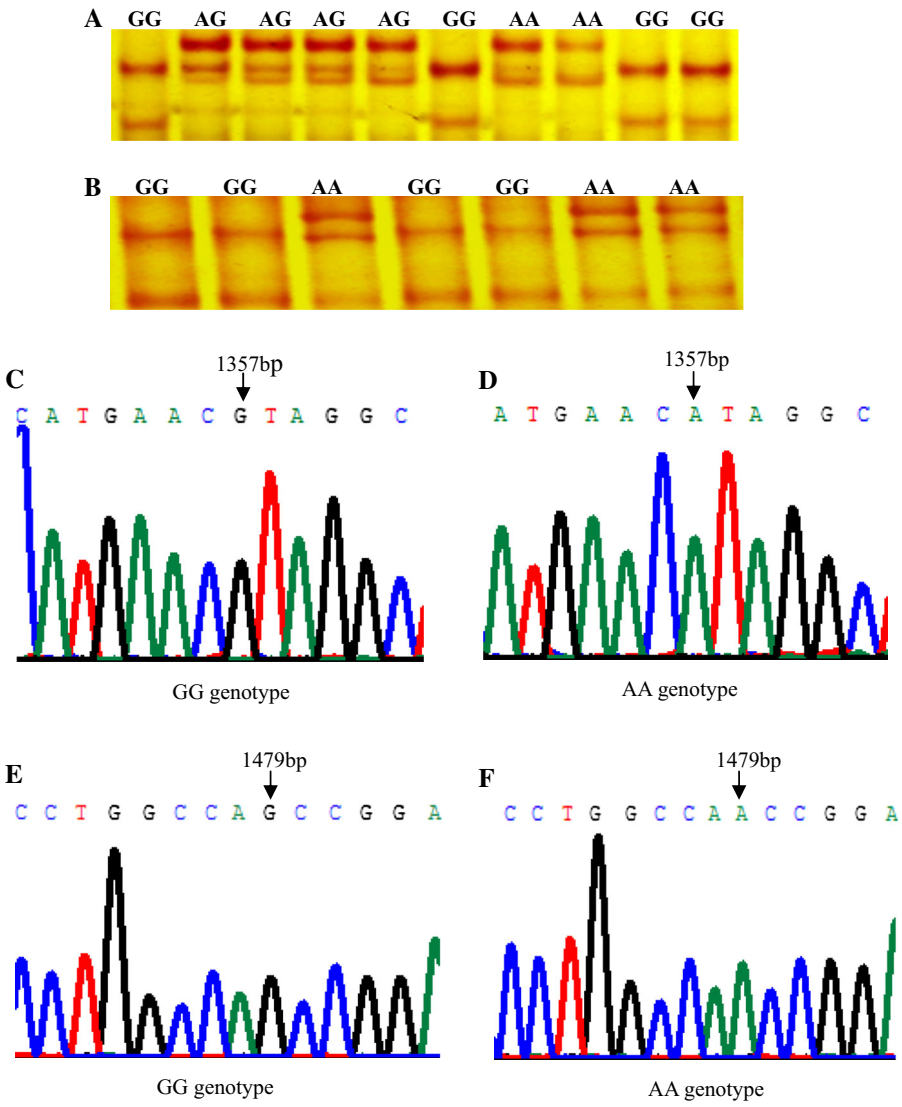


Fig. 1 *GHR1* gene genotyping and sequencing identify. **a** Band pattern for genotypes (GG and AA) of L1 locus in exon 8; **b** band pattern for genotypes (GG and AA) of L2 locus in exon 9. The chromatograms show sequences of the two SNPs (c.G1357A and c.G1479A): The number indicates the position of mutation site; **c** sequence of GG genotype in exon 8; **d** sequence of AA genotype in exon 8; **e** sequence of GG genotype in exon 9; **f** sequence of AA genotype in exon 9

Table 2 Association between SNPs of *GHR1* gene and measured traits by ANOVA

Traits locus	T3 (ng/ml)	T4 (μg/dl)	IGF (ng/ml)	Length (cm)	Weight (g)	Weight without viscera (g)	Liver weight (g)	Gonad weight (g)	HSI	GSI
L1	*	NS	NS	NS	**	NS	NS	*	NS	NS
L2	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS

* $P \leq 0.05$; ** $P < 0.01$; *NS* no significantly

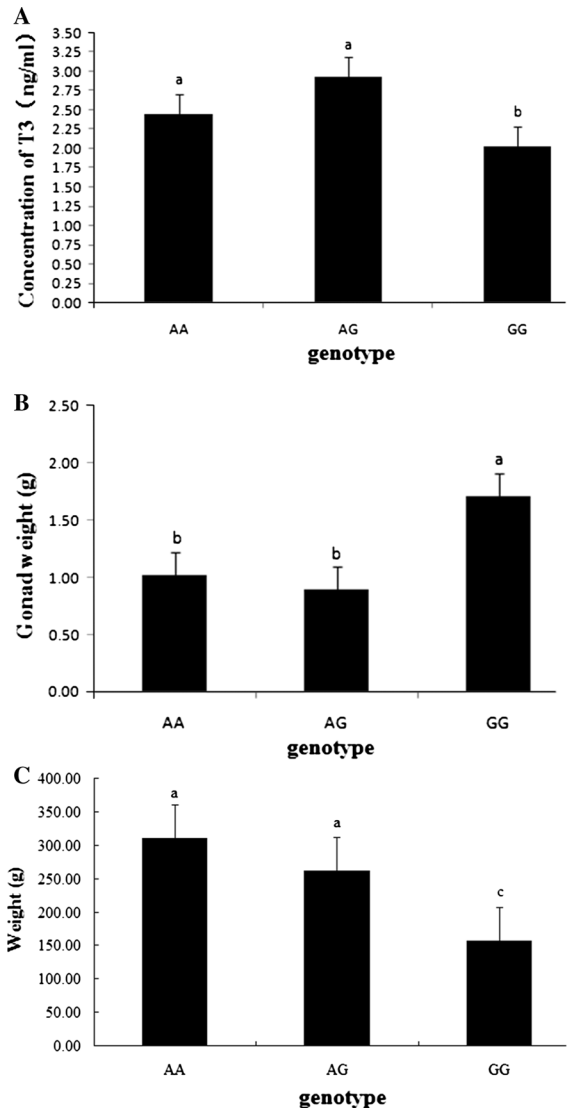


Fig. 2 Multiple comparisons of growth traits and hormone level among different genotypes of L1 locus. **a** T3 level of AA, AG and GG genotype in L1; **b** gonad weight of AA, AG and GG; **c** weight of AA, AG and GG. Different *letters* above the *error bars* mean significant difference at $P \leq 0.05$ and $P < 0.01$

weights of AA (310.6 ± 4.4217 g), AG (261.9 ± 1.6713 g) and GG genotypes (156.3 ± 5.7085 g; Fig. 2c). L2 locus had no significant association with measured traits ($P > 0.05$).

DNA methylation patterns

Three CpG dinucleotide sites were found in the amplification of exon 8 of half-smooth tongue sole in GG genotype, which was due to the mutation from A to G at nucleotide 1357 bp in exon 8 (Fig. 3). Two CpG dinucleotide sites were found in AA and AG genotypes. The new CpG site in GG genotype was highly methylated (93.3 %). The other two CpG sites were also highly methylated (≥93.3 %), but there were no difference among three genotypes. In addition, in the thirty individuals of AG heterozygous genotype, two of them were found CpG site at 1357 bp (Fig. 4).

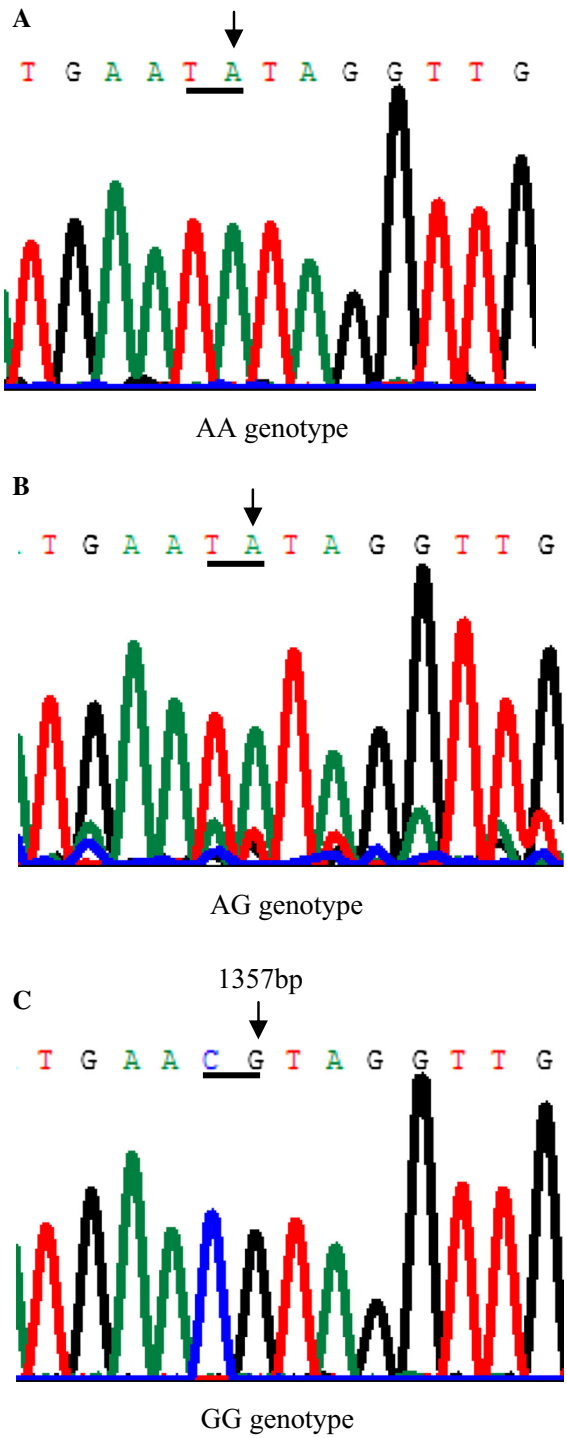
GHR1 gene expression level

GHR1 gene and β-actin had a nice linear relationship within the concentration range. The correlation analysis showed that there was highly significant association between L1 locus and *GHR1* gene mRNA expression (Table 3). Further multiple comparisons demonstrated that the GG genotype had lower gene expression level ($P < 0.05$) than AA and AG genotypes, and there was no significant difference between AA and AG genotypes (Fig. 5).

Discussion

Mutations of gene exons could influence some traits in human and animals. Previous studies reported that coding variants in exons of human *ASMT* gene might be associated with autism risk (Wang et al. 2013). Polymorphisms in exons 4 and 5 detected in the goat *GH* gene showed a beneficial effect on milk production (Malveiro et al. 2011). In pig population, two SNPs in exons 3 and 5 of *IL10* gene were indentified and were significantly associated with serum IL10 level, a kind of cytokines with anti-inflammatory properties (Liu et al. 2012). Polymorphism of exon 2 of *GH* gene may have impact on the production performance in Huoyan goose (Zhang et al. 2014). Similarly, in the present study, polymorphisms of half-smooth tongue sole *GHR1* gene were identified and were associated with hormone level and growth traits. We found that there were three genotypes, AA, AG and GG, in half-smooth tongue sole due to the L1 locus mutations of *GHR1* gene exon 8 and two genotypes AA and GG according to the L2 locus mutation of exon 9.

The growth hormone receptor (GHR) is required for GH to carry out its effect. *GHR* gene regulates the synthesis and secretion of GHR. The mutation of *GHR*



◀**Fig. 3** Amplified fragments from bisulfite treatment DNA. The *number* shows the position of mutation site: **a** The cytosine close to adenine was converted to thymine in AA genotype; **b** sequence of AG genotype (double crest); **c** there was a novel CpG site dinucleotides due to transition between guanine and adenine at 1357-bp position. The cytosine was methylated in GG genotype

gene may affect the number and structure of GHR, which will influence its biological function and growth performance, eventually. Many studies were about *GHR* gene polymorphisms and its association with growth traits. Sharma et al. (2013) reported that one SNP of *GHR* gene was found to be associated with body weight at birth in Jamunapari breed of goat. In humans, mutations in the *GHR* gene have been associated with Laron-type dwarfism (Godowski et al. 1989). Ni et al. (2008) reported that microsatellite polymorphisms of GHR promoter were significantly associated with some growth traits in *Paralichthys olivaceus*. There were five SNPs in *C. carpio* var. Jian associated with weight gain significantly (Tao et al. 2011). These indicated that it was necessary to study GHR gene. These results proved that mutations in *GHR* gene could influence the growth traits. In the present study, mutations of A and G nucleotide were indentified in half-smooth tongue sole. L1 site was associated with growth traits. The weight of AA genotype and AG genotype was higher than GG genotype, which indicated that A allele had more advantages over G allele. What's more, G allele mutation in *GHR1* gene down-regulated the growth of the individuals of GG genotype in half-smooth tongue sole. Bahrami et al. (2013) found some SNPs in *GH*, *IGF-I* and *LEP* gene of Mehraban sheep, which lead to the substitution of amino acids, and then have an influence on protein structure and gene biological functions. The result in our study showed that the mutation of L1 was a non-synonymous mutation, which resulted in the change of amino acid. The mutation caused Val (376) to Ile (376) transition. This might affect the protein structure of GHR, which could decrease the binding efficiency of GH–GHR. The decreased combination rate could have a negative effect on the promotion function of GH and lead to slow growth. But the protein activities should be testified by further experiment.

Interestingly, L1 locus was associated with T3 level. In vertebrates, thyroid hormone plays important roles in regulating development, differentiation and metabolism and most vertebrates are unable to grow and reach their normal adult form without them (Porterfield and Henderson 1993). Triiodothyronine (T3) is the biologically active form of thyroid

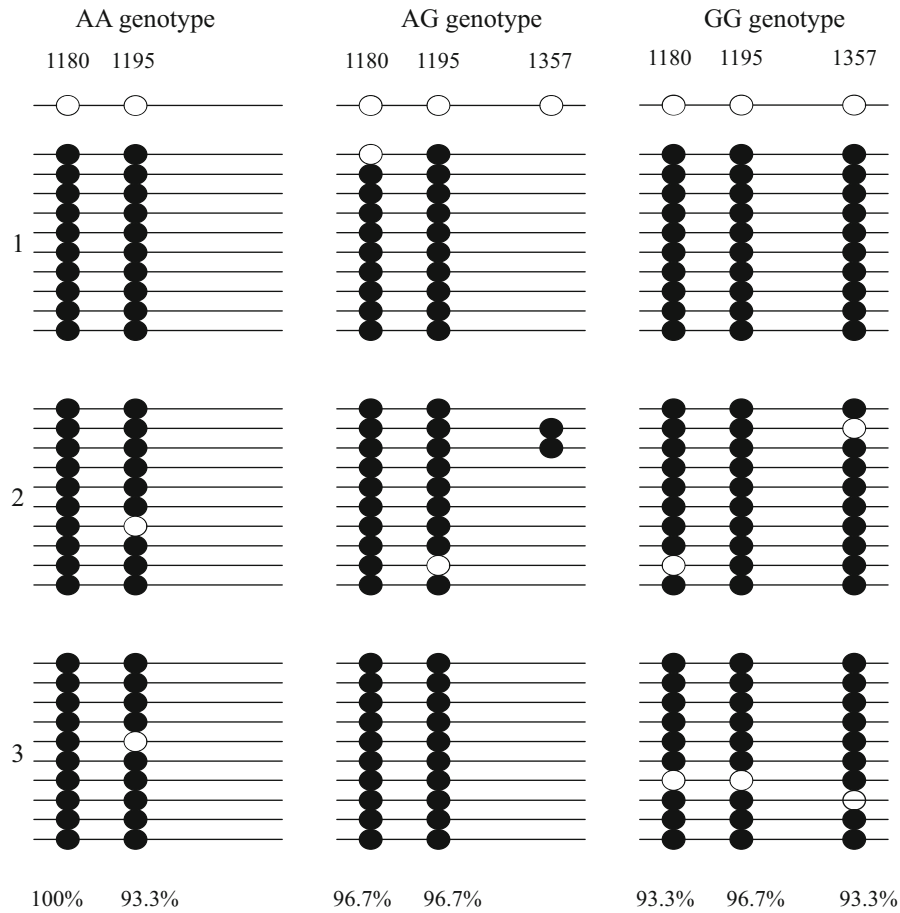


Fig. 4 DNA methylation patterns of exon 8 in *GHR1*. *Filled* and *open circles* denote methylation and unmethylation sites, respectively. Each *line* represents one sequenced clone. The *first line* indicates the localizations of studied CpG site related to the sequence of *GHR* coding region. No. *1*, *2*, *3* refer to individual fish from each genotype. The *percentage* indicates the methylation level of each CpG site (The *number under* the *line*)

Table 3 Association between SNPs and *GHR1* gene expression by ANOVA

Locus	*GHR1* gene expression	
	F value	*P* value
L1	19.77	0.0005**

** $P < 0.01$

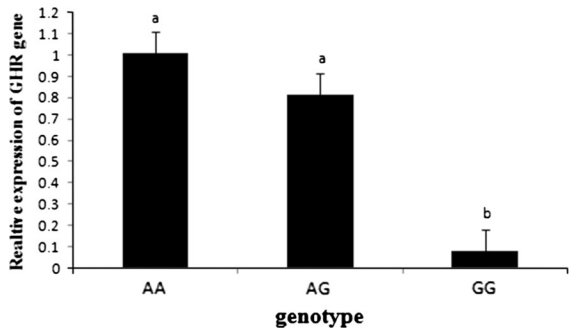


Fig. 5 Expression levels of *GHR1* gene of different genotypes. *Different letters above the error bars* mean significant difference at $P < 0.01$

hormone. The addition of T3, along or together with GH, stimulates *GHR* mRNA expression in hepatocytes of adult male rats (Tollet et al. 1990). There were studies about thyroid hormone promoting the growth in fish species. Thyroid hormones are thought to act synergistically with GH to regulate growth and nutrient partitioning in teleosts, with T3 directly stimulating production of growth hormone releasing hormone receptor in the pituitary (Korytko and Cuttler 1997). In common with observation in mammals and birds, thyroid hormone was also shown to be an important factor in the developmental transition of muscle protein isoforms such as myosin in Japanese flounder (Yamano et al. 1994). Schmid et al. (2003)

reported that thyroid hormone stimulates hepatic *IGF-I* mRNA expression in the tilapia (*Oreochromis mossambicus*). These indicated that the thyroid hormone up-regulates the growth and T3 could affect the expression of *GHR* gene. The expression level of *GHR* gene related directly to the number of GHR. In this study, we deduced that T3 increased the *GHR1* expression level and promoted their growth in AA and AG genotypes compared with GG genotype of male half-smooth tongue sole. There was another discovery that serum IGF-I had no significant association with genotypes in L1 locus. *IGF-1* mRNA was mainly expressed in liver, and serum IGF-I performed its function through growth hormone. There maybe some relationship between GHR1 and IGF-1, but that is not the case in our study. In blunt snout bream, the development-related *GHR1* was positively correlated with *IGF-I*, while the tissue-related *GHR1* had no significant correlation with *IGF-I*. So we deduced that relationship between *GHR1* and IGF-1 was time dependent and tissue specific according to the existing research (Zeng et al. 2014). We need to do further study to understand how GHR effect on IGF-I.

Further experiment in this study testified differences in *GHR1* gene expression level among three genotypes of half-smooth tongue sole. Many studies have reported that the distribution of fish *GHR* mRNA was mainly in liver, for example, gilthead sea bream (*Sparus aurata*; Calduch-Giner et al. 2003) and Japanese eel (*Anguilla Japonica*; Ozaki et al. 2006). So the extraction of RNA was performed in liver tissue. The result of quantitative real-time PCR showed that AA and AG genotypes had higher *GHR1* expression level than GG genotype (Fig. 5). Many researches show the correlation between *GHR* mRNA expression and growth. Abruptly elevated level of hepatic *GHR* mRNA in chicken accompanying the highest growth rate exhibited during first 3 weeks after hatching (Zhao et al. 2004). The decline in *GHR* mRNA expression was found in fasted coho salmon, aea bream and eel, inducing the decrease in the number of GHR, binding rate of GH with GHR, and IGF-I synthesis in liver. These were all important factors of the decline of GH growth-promoting effect (Saera-Vila et al. 2005). Fukada et al. (2004) showed that GHR gene expression in salmon reduced following a 3-week fast, the binding rate of GH to GHR decreased, and the growth-promoting function of GH was weaken and then slowed down fish growth. Higher expression level contributed to an increase in the number of GHR, which meant there was more possible for GH to combine with. We concluded that AA and AG genotypes had a nice growth status than GG genotype. Many factors could affect the gene expression. The influential factors of gene expression included not only hormone, but also DNA methylation level.

As a kind of epigenetic modification, DNA methylation refers to the transfer of a methyl group to the carbon-5 position of cytosine and occurs in cytosine–phosphodiester–guanine (CpG) nucleotides. Generally speaking, DNA methylation could repress the transcriptional activity and demethylation might associate with gene activity. There were many studies showed that DNA methylation was an important inhibitor of gene expression. It was reported that in mammals, *fads*2 gene expression in the liver has shown to be down-regulated by the hypermethylation of CpG sites in the promoter (Devlin et al. 2005). There was report demonstrated that in bovine, *cyp17A1b* and *cyp17A1x* were both silenced by promoter methylation (Vanselow and Fürbass 2011). In Japanese flounder, highly methylation CpG site caused lower gene expression level of *cyp17-II* (Ding et al. 2012), and in Nile tilapia, the methylation of *GH* promoter was negatively correlated with *GH* mRNA expression in pituitary and the growth rate (Zhong et al. 2014). In our study, due to the transition from A to G, there was a new CpG site in GG genotype (Fig. 4). There were three CpG sites in GG genotype and two CpG sites in AA and AG genotypes. The newly created CpG was located at 1357 bp and was highly methylated (93.3 %) in GG genotype, while CpG sites at 1180 and 1195 bp were also high methylated in all genotypes and there was no difference among them. According to the methylation state, we proposed that AA and AG genotypes had higher *GHR1* mRNA expression than GG genotype and the proposition was confirmed by the quantitative real-time PCR (Fig. 6). Gene expression was silenced by CpG methylation in GG genotype of half-smooth tongue sole in some extent. If we could remove the methylation of *GHR1* gene and test whether the gene expression could be restored, the result would be more convictive. Although two of thirty individuals of AG genotype were found 1357 bp CpG site, there were no difference between AA and AG genotypes at the aspect of hormone level, growth traits and gene

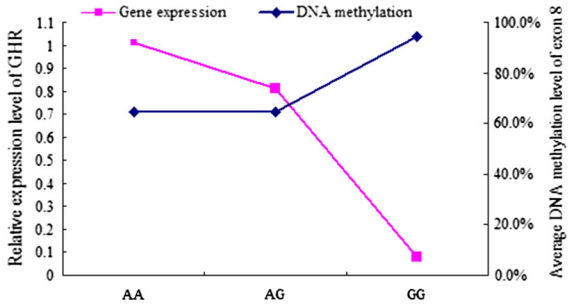


Fig. 6 The correlations between gene expression and average CpG methylation level of *GHR1* gene of different genotypes

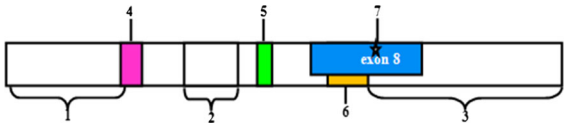


Fig. 7 The mutation location of exon 8 in the *GHR1* structure: (*1*) ligand-binding domain; (*2*) transmembrane domain; (*3*) intracellular domain; (*4*) WSXWS-like motir; (*5*) Box1; (*6*) Box2; and (*7*) L1 locus

expression level. Therefore, we speculated that epigenetic modification of L1 locus could regulate the *GHR1* gene transcription and L1 locus could be used as a potential genetic or epigenetic marker for half-smooth tongue sole. What's more, through comparison with the *GHR1* gene structure, we fixed the position of exon 8 and the mutation site (Fig. 7). The transition (c.G1357A) occurred in the intracellular domain and between Box2 and the second tyrosine residues. As to the mutation occurred near by the Box2 and the tyrosine residues, whether it had influence on their function being a question that can be discussed in further researches. It was necessary to performed further experiments on other generations to further testify the speculation.

Conclusions

In this study, we identified two SNPs in *GHR1* gene of half-smooth tongue sole and just one of them had association with growth traits and T3 level. Individuals could be divided into three genotypes, AA, AG and GG based on L1 locus. This mutation located in CpG site of *GHR1* gene. GG genotype had lower weight, and T3 level and gene expression level compared with AA and AG genotypes. These results provided theoretical evidences for clarifying the mechanism of half-smooth tongue sole GHR1 physiology. What's more, AA and AG genotypes of L1 locus could be a potential candidate genetic and epigenetic marker in half-smooth tongue sole breeding programs in finding well-grown male individuals for market and scientific research.

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