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Short Communication

Polymorphism in *GHRH* Gene and Its Association with Growth Traits in Tibetan Sheep

Lin-Sheng GUI [1,3,†] Yang-Yang JIANG [3,†] Gui-Yao LIU [2,†] Jian-Lei JIA [3]
Ming-De CHU [4] Sheng-Zhen HOU [1,3] Zhi-You WANG [1,3]

[†] *These authors contributed equally to this study*
[1] State Key Laboratory of Plateau Ecology and Agriculture, Qinghai University, Xining, Qinghai Province 810016, P. R. CHINA
[2] Poyang Lake Eco-economy Research Center, Jiujiang University, Jiujiang, Jiangxi Province, 332005, P. R. CHINA
[3] College of Agriculture and Animal Husbandry, Qinghai University, Xining, Qinghai Province 810016, P. R. CHINA
[4] Animal Husbandry and Veterinary Station, Menyuan county, Qinghai province, 810016, P. R. CHINA

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Abstract

The objectives of this study were to identify single nucleotide polymorphisms (SNPs) in the growth hormone-releasing hormone *(GHRH)* gene and to evaluate their associations with growth traits in two main Tibetan sheep breeds. Through sequencing technology, four SNPs were identified in the 5'UTR region of *GHRH* gene. Both g.794A>C and g.1497C>A exhibited significant influence on the growth-related traits in Tibetan sheep (P<0.05 or P<0.01). Hence, the biochemical and physiological functions, together with the results obtained in our investigation, suggest that the *GHRH* gene could serve as genetic marker for growth in Tibetan sheep breeding.

Keywords: *Tibetan sheep, Mutation, GHRH gene, Body measurement*

Tibet Koyunlarında Büyüme Hormonunu Salgılatıcı Hormon *(GHRH)* Genindeki Polimorfizmlerin Büyüme Özellikleri İle İlişkisi

Öz

Bu çalışmanın amacı, büyüme hormonu salgılatıcı hormon *(GHRH)* genindeki tek nükleotid polimorfizmlerini (SNPs) tanımlamak ve bunların iki Tibet koyun ırkının büyüme özellikleri ile olan ilişkilerini değerlendirmektir. Sıralama teknolojisi ile, *GHRH* geninin 5'UTR bölgesinde dört SNP tanımlandı. Hem g.794A> C hem de g.1497C> A, Tibet koyunlarında büyümeyle ilgili özellikler üzerinde önemli bir etki gösterdi (P<0.05 veya P<0.01). Bundan dolayı, araştırmamızda elde edilen sonuçlarla birlikte, biyokimyasal ve fizyolojik fonksiyonlar *GHRH* geninin Tibet koyun yetiştiriciliğinde büyüme için genetik belirteç görevi görebileceğini ortaya koydu.

Anahtar sözcükler: *Tibet koyunu, Mutasyon, GHRH geni, Vücut ölçüsü*

INTRODUCTION

Tibetan sheep (ovine) were the first artificially bred sheep in the Qinghai-Tibetan plateau and hold enormous potential for animal production [1], which showed high tolerance to the extreme environments, such as extreme cold, low oxygen concentrations, and low air pressure [2]. As an anabolic hormone, growth hormone *(GH)* was synthesized and secreted by the anterior pituitary eosinophil cells in mammals [3]. It could perform crucial effect on tissue growth, reproduction, muscle accretion and fat catabolism by binding to various hormones of the somatotropic axis [4]. In the anterior pituitary gland and tissues, the synthesis and secretion of *GH* was highly affected by *GHRH* [5,6]. Because of the specific role of the *GHRH* gene in metabolism, we hypothesized that the variations in *GHRH* gene would be a candidate for heritable differences in growth traits of Tibetan sheep.

MATERIAL and METHODS

Blood samples were obtained from 565 female Tibetan sheep (aged 6 to 8 months) belonging to two different breeds: Black Tibetan sheep (BT, N=210) and Oula Tibetan

İletişim (Correspondence)
+86-09715318030; Fax: +86-09715318030
wangzhiyou52@163.com

sheep (OT, N=355). The sheep were reared in Henan County and Haiyan County respectively of Qinghai Province. In this study, all individuals were raised in five different farms. Meanwhile, the growth traits including body weight, withers height, body length and heart girth were measured on one day. Genomic DNA were isolated from whole blood samples using the OMGAM Blood DNA Kit (OMGAM Bio-Tek, Doraville, USA) and were stored at -20°C. On the basis of the *GHRH* gene sequence (Accession no NC_019470), four pairs of PCR primers *(Table 1)*. were designed by Primer Premier Software (Version 5.0). PCR amplification was performed following the method of Sun et al.[7] Amplification were then sequenced using an ABI 3730 sequencer (ABI, Foster City, CA, USA).

Gene frequencies, Hardy-Weinberg equilibrium and polymorphism information content were calculated by POPGENE software package (Version 3.2). Linkage disequilibrium (LD) was estimated by the web-based tool *(http://analysis.bio-x.cn/myAnalysis.php)*. SNPs interaction was assessed based on likelihood ratio statistic test from logistic regression [8]. Statistical analysis was performed using the general linear model (GLM) procedure implemented in SPSS 16.0 (IBM Company, NY, USA) software package. The basic linear model was: $Y_{ijk} = \mu + G_i + A_j + F_k + e_{ijk}$, G_i is the fixed effect of genotype, A_j is the fixed effect of age, F_k is the fixed effect of farm and e_{ijk} is the random error.

RESULTS

Four SNPs were identified in the 5'UTR of ovine *GHRH* gene including g.794A>C, g.987T>C, g.1480G>A and g.1497C>A *(Fig. 1)*. Summary statistics for each of those SNPs were presented in *Table 2*. The frequencies of allele A (g.794A>C), T (g.987T>C), G (g.1480G>A) and C (g.1497C>A) were found to be predominant in the studied samples. Except for g.987T>C in OT breed and BT breed, and g.1497C>A in OT breed, the genotypic frequencies conformed to Hardy-Weinberg equilibrium (P>0.05). According to the classification of PIC value [9], four SNPs were within the range of moderate genetic diversity (0.25<P<0.50). The values of r^2 between the four SNPs in the studied samples were from 0.000 to 0.043, indicating that those SNPs had weak LD.

The results of association study between BT breed and growth traits were presented in *Table 3*. For the g.794A>C and g.1497C>A, the influence of AA genotype resulted in the highest mean for body weight compared to animals with genotype CC (P<0.05). As shown in *Table 4*, the g.1497C>A polymorphism affected the body weight, and heart girth in animals with the AA genotype to a much greater extent than in animals with the CC genotypes (P<0.01 or P<0.05) in OT breed.

The prediction indicated that substituting A with C at g.794A>C locus produced a putative loss of binding sites myf3, bHLH transcription factor 1, TGFB-induced factor homeobox 2-like and myf4. In addition, the prediction suggested that g. 1497 C and g. 1497 A could in sequence binding in six and four cis-acting elements *(Table 5)*.

As it is presented in *Table 6*, an examination of the epistatic effect of the three-gene SNP genotypes on the growth traits was attempted, with significant interactions detected for four growth traits (body weight, withers height, body length and heart girth).

DISCUSSION

Our results were consistent with the previous study. Piorkowska et al.[10] reported that the *GHRH/AluI* SNP had significant effects on water-holding capacity and meat colour in pigs of three breeds reared in Poland. One novel SNP *(GHRH*/HealII) was demonstrated to improve body

Table 1. *Primers used in these experiments*

Name	Primer Sequence (5' to 3')	Tm (°C)	Product Length	Amplified Region
L1	CGTCAGTGCTTTAGGGTTC	58.8	695 bp	Part of 5'UTR
	GATTGGCAGATTTGGGAG			
L2	CTGGCTTTACTGCGACTT	60.0	550 bp	Part of 5'UTR
	TGGCATTCTACTCCCTCC			
L3	GTGACTGGCAGAGGCAGA	61.5	760 bp	exon 1 and exon 2
	GAAGTGACAGCTGCTGTG			
L4	AAAGGGCAGTTCTTCATA	63.5	771 bp	exon 3
	TCTTCTGGTTCTTGATGTAT			

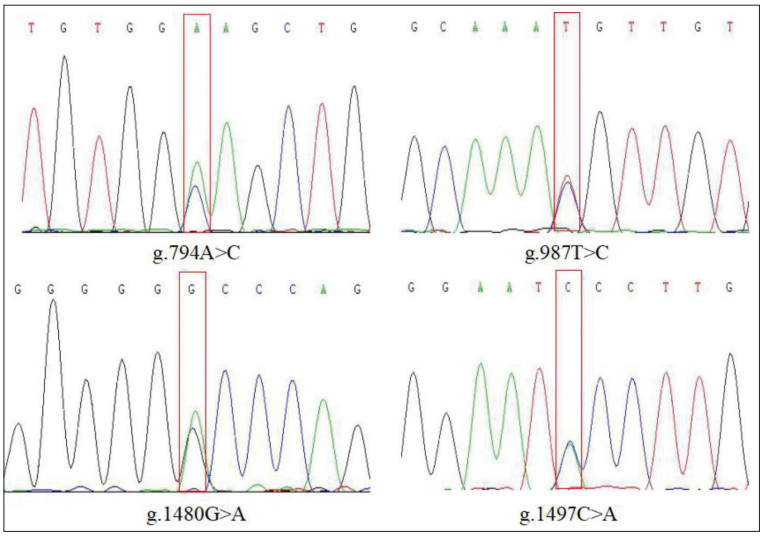

Fig 1. The sequencing map of the GHRH gene SNPs

Table 2. *Genotype frequencies (%) of the GHRH gene for the single nucleotide polymorphisms (SNPs)*

Locus	Breeds	Genotypic Frequency (%)			PIC	Maximum Allele Frequency	HWE
g.794A>C	BT	AA (48.57)	AC (37.14)	CC (14.29)	0.34	67.14% (A)	5.26
	OT	AA (56.62)	AC (36.06)	CC (7.32)	0.31	74.65% (A)	0.80
g.987T>C	BT	TT (61.90)	TC (27.14)	CC (10.95)	0.30	75.48% (T)	14.95
	OT	TT (72.11)	TC (19.15)	CC (8.73)	0.26	81.69% (T)	45.92
g.1480G>A	BT	GG (59.52)	GA (34.29)	AA (6.19)	0.29	76.67% (G)	0.37
	OT	GG (46.48)	GA (41.97)	AA (11.55)	0.34	67.46% (G)	0.68
g.1497C>A	BT	CC (52.86)	CA (37.62)	AA (9.52)	0.32	71.67% (C)	1.13
	OT	CC (67.04)	CA (24.51)	AA (8.45)	0.31	79.30% (C)	22.83

Polymorphism information content (PIC), heterozygosity (He), Hardy-Weinberg equilibrium (HWE), $\chi^2_{0.05(1)} = 3.840$, $\chi^2_{0.01(1)} = 6.630$

Table 3. *Association of different genotypes of SNPs in GHRH gene with growth traits in BT breed*

Locus	Genotypes (N)	Body Weight (kg)	Withers Height (cm)	Body Length (cm)	Heart Girth (cm)
g.794A>C	AA (102)	47.60 ± 0.26^{a}	67.90±0.24	71.00±0.24	90.78±0.41
	AC (78)	44.56 ± 0.30^{ab}	67.05±0.28	70.13±0.27	91.47±0.47
	CC (30)	43.08 ± 0.47^{b}	66.37±0.45	69.82±0.43	90.02±0.76
g.987T>C	TT (130)	45.29±0.27	67.40±0.22	70.57±0.21	90.49±0.37
	TC (57)	46.21±0.41	67.65±0.33	70.40±0.32	91.71±0.55
	CC (23)	47.87±0.65	66.38±0.53	70.43±0.51	91.48±0.68
g.1480G>A	GG (126)	45.86±0.28	65.54±0.22	70.22±0.21	90.98±0.31
	GA (72)	45.78±0.37	67.21±0.29	71.07±0.28	90.84±0.49
	AA (12)	45.74±0.63	66.74±0.52	70.16±0.59	90.85±0.69
g.1497C>A	CC (111)	44.64 ± 0.28^{b}	67.00±0.23	69.97±0.21	90.54±0.39
	CA (79)	46.80 ± 0.32^{ab}	67.57±0.27	70.68±0.26	91.45±0.47
	AA (20)	48.83 ± 0.55^{a}	68.74±0.46	72.51±0.45	90.99±0.84

^{a,b} Means with different superscripts are significantly different (P<0.05); ^{A,B} Means with different superscripts are significantly different (P<0.01)

Table 4. *Association of different genotypes of SNPs in GHRH gene with growth traits in OT breed*

Locus	Genotypes (N)	Body Weight (kg)	Withers Height (cm)	Body Length (cm)	Heart Girth (cm)
g.794A>C	AA (201)	58.87 ± 0.35^{a}	71.60±0.26	75.29 ± 0.26^{a}	96.34 ± 0.33^{a}
	AC (128)	58.49 ± 0.44^{a}	71.20±0.31	74.80 ± 0.32^{ab}	95.82 ± 0.42^{a}
	CC (26)	52.40 ± 0.73^{b}	70.00±0.53	71.95 ± 0.47^{b}	92.12 ± 0.64^{b}
g.987T>C	TT (256)	57.69±0.32	71.16±0.24	74.58±0.23	95.77±0.30
	TC (68)	58.78±0.62	71.35±0.45	75.12±0.45	95.84±0.58
	CC (31)	61.83±0.75	72.78±0.67	76.69±0.66	96.40±0.70
g.1480G>A	GG (165)	58.71 ± 0.39^{a}	71.32±0.29	74.93±0.28	95.91±0.37
	GA (149)	58.97 ± 0.43^{a}	71.68±0.31	75.59±0.29	96.37±0.39
	AA (41)	53.89 ± 0.71^{b}	70.19±0.58	72.03±0.56	93.62±0.74
g.1497C>A	CC (238)	57.99 ± 0.31^{B}	70.99±0.24	74.71 ± 0.28^{b}	95.94±0.31
	CA (87)	56.55 ± 0.51^{B}	71.12±0.39	73.94 ± 0.38^{b}	94.92±0.51
	AA (30)	65.29 ± 0.82^{A}	74.77±0.66	78.89 ± 0.64^{a}	97.76±0.80

measurement in limousine cattle [11]. *Cheong* et al.[12] showed that one SNP (c.-4241A>T) in promoter region of *GHRH* gene had a strong effect on cold carcass weight and longissimus muscle area in Korean Hanwoo cattle. The research of Zhang et al.[13] showed that a novel marker (g.4251C>T) on *GHRH* gene was associated with body weight for different growth periods (6, 12, 18, and 24 months old) in Nanyang cattle. Based on the outcomes, it is our belief that the *GHRH* gene could be an excellent candidate gene for growth-related traits in livestock.

Table 5. *Transcription factor binding sites identified at SNP's identified within 5'UTR of ovine GHRH gene*

Locus	Genotype	Transcription Factors	Core Similarity	Cis-acting Elements (Recognition sequence)	Target Strand
g.794A>C	A/C	Transcription factor AP-2, alpha	0.952	tggcCCTGtgga/cagc	(+)
	A	CCAAT/enhancer binding protein beta	0.928	ggcccTGTGgaagct	(+)
	C	TGFB-induced factor homeobox 2-like, X-linked, dimeric binding site	0.925	cagacagctGCCAcagg	(-)
	C	Myogenic regulatory factor MyoD (myf3)	0.977	ctgtggCAGCtgtctgc	(+)
	C	Achaete-scute family bHLH transcription factor 1	0.978	cagacaGCTGccaca	(-)
	C	Myogenic bHLH protein myogenin (myf4)	0.992	ggcagaCAGCtgccaca	(-)
	C	TALE homeobox protein Meis 2, dimeric binding site	0.779	gtggCAGCtgtctgcca	(+)
g.1497C>A	C/A	PAX6 paired domain and homeodomain are required for binding to this site	0.880	Atc/accttgtgCCAGccctg	(+)
	C/A	Stimulating protein 1, ubiquitous zinc finger transcription factor	0.885	CccagGGGCtgggaatc/a	(+)
	C	Ikaros 1, potential regulator of lymphocyte differentiation	0.947	ggctGGGAatccc	(+)
	C	NF-kappaB (p50)	0.851	gctGGGAatcccttg	(+)
	C	SRY (sex-determining region Y) box 9	0.968	ctggcACAAgggattcccagccc	(-)
	C	Transcription factor with 8 central zinc fingers and an N-terminal KRAB domain	0.772	agggggggCCAGgggctgggaatcc	(+)
	A	Ikaros 3, potential regulator of lymphocyte differentiation	0.997	ggctgGGAAtacc	(+)
	A	Brn-5, POU-VI protein class (also known as emb and CNS-1)	0.754	ggctggCACAaggtattcccagc	(-)

Table 6. *Multi-marker interaction analysis of GHRH with GH and Myogenin*

Traits	Gene	df	Sum of Square	Mean of Square	F Value	P Value
Body length	GHRH	2	309.1	154.54	15.371	6.39E-07
	GH	2	170.3	85.16	8.47	0.000298
	MY	2	259.7	129.83	12.914	5.45E-06
	GHRH:GH	4	27.9	6.97	0.694	0.59711
	GHRH:MY	3	22.9	7.62	0.758	0.519159
	GH:MY	4	19.9	4.97	0.494	0.740071
	GHRH:GH:MY	4	43.7	10.92	1.086	0.364838
Body weight	GHRH	2	910.8	455.4	40.089	2.75E-15
	GH	2	308.9	154.5	13.597	2.99E-06
	MY	2	485.4	242.7	21.362	4.16E-09
	GHRH:GH	4	11.4	2.8	0.251	0.909
	GHRH:MY	3	49.6	16.5	1.454	0.228
	GH:MY	4	4.1	1	0.089	0.986
	GHRH:GH:MY	4	26.2	6.6	0.577	0.680
Heart girth	GHRH	1	2405	2405	0.581	0.447
	GH	2	8532	4266	1.03	0.359
	MY	2	3275	1637	0.395	0.674
	GHRH:GH	2	5169	2585	0.624	0.537
	GHRH:MY	2	2110	1055	0.255	0.775
	GH:MY	4	8878	2220	0.536	0.709
	GHRH:GH:MY	4	4506	1126	0.272	0.896
Withers height	GHRH	2	502.2	251.1	17.596	9.62E-08
	GH	2	60.9	30.46	2.134	0.12114
	MY	2	168.9	84.44	5.917	0.00321
	GHRH:GH	4	79.5	19.88	1.393	0.23794
	GHRH:MY	3	45.5	15.18	1.064	0.36561
	GH:MY	4	56.4	14.09	0.988	0.41543
	GHRH:GH:MY	5	136.3	27.25	1.91	0.09446

Silico analysis was used to predict the effects of the alternative alleles in the 5′ UTR of the *GHRH* gene on the transcription factor binding sites by online analysis website *(http://www.genomatix.de/)* [14]. The predictable results showed that differences existed for the transcription factors of the different genotypes at these two mutations. Thus, it can be reasonably inferred that the identified SNPs within the *GHRH* gene 5′UTR regions would modify transcription factor binding affinity, thereby affecting phenotypes in Tibetan sheep.

Our previous work revealed that SNPs of candidate (i.e., *GH* and *Myogenin*) significantly influenced growth traits in Tibetan sheep [7,15]. Multi-marker interaction analysis suggested the influence of the mutations in three different genes were additive effects, which is not consistent with the the function of *GHRH* in regulating the *GH* secretion. This discordance may be due to relatively small sample size used. Further studies are required to address other SNPs of the three genes and their associated gene network.

In summary, four polymorphisms in the *GHRH* gene were identified in Tibetan sheep. The association analysis of single markers revealed that g.794A>C and g.1497C>A exhibited prominent effects on growth traits. Our investigation provides evidence that *GHRH* gene could be used as molecular markers and could contribute to the expanding panel of functional variation.

Conflicts of Interest

The authors declare no conflict of interest.

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